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THE EPIDEMIOLOGY OF NECATOR AMERICANUS INFECTION IN RURAL THAILAND

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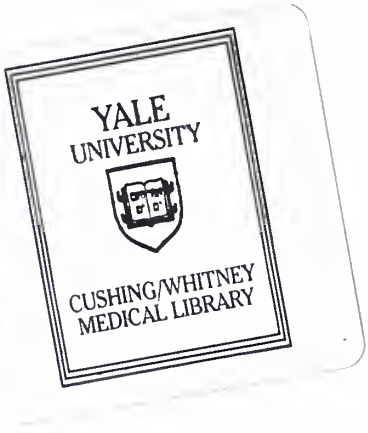
1997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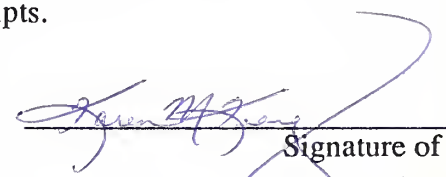

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The Epidemiology of *Necator americanus* Infection in Rural Thailand

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

Karen Minnie Kiang

1997

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THE EPIDEMIOLOGY OF *NECATOR AMERICANUS* INFECTION IN RURAL THAILAND.

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We determined the prevalence, intensity, and distribution of human hookworm infection (as well as degree of co-infection by *Ascaris lumbricoides* and *Trichuris trichiura*) along the central Thai-Myanmar border. 660 individuals from 3 villages underwent stool examination for fecal egg counts using the McMaster's counting technique. Age of participants ranged from 0-85 years old. Hookworm larvae were cultured and brought back to the United States for species determination using a PCR-RFLP technique. Using PCR-RFLP, the predominant species was found to be *Necator americanus*, with very little if any evidence for *Ancylostoma duodenale* in our population. Hookworm distribution was highly aggregated, with 65.8% of those infected having light infections (<1000 eggs per gram stool). On the other end of the intensity spectrum, 14.8% of infected subjects accounted for 55.8% of the total hookworm egg production. Overall hookworm prevalence was found to be 57.2%. Prevalence and mean intensity varied co-linearly and showed a strong age-dependence. They climbed steadily throughout childhood and adolescence before plateauing in the 26-30 year old age group, where they were maintained through to the oldest age group. They did not increase or decrease with age. Hookworm levels also varied by village, with Village 2 showing significantly lower prevalence and mean intensities of *Necator* than Villages 1 and 3. Sex was not a statistically significant factor.

Co-infection by *Ascaris* and *Trichuris* was rare. Overall *Ascaris* prevalence was 6.4% (data for Trichuris was unavailablee). Only 5.8% of those infected with *Necator* were co-infected with *Ascaris*. Ethnically, the Mon group had a higher risk of being infected than the Karen group, and were infected at higher levels. However, since Village 2 is made up of mostly Karen people and Villages 1 and 3 by Mon people, it is impossible to distinguish between "village" and "ethnic group" as separate risk factors. Other risk factors proved to be both the lack of a latrine, and the types of bathroom floor. Dirt and bamboo floors carried a higher risk for infection, while cement floors were protective. Shoe-wearing as a risk factor was eliminated during statistical analysis due to reporting inaccuracies. In comparing the frequency of hookworm-infected mother-infant pairs to pairs in which one or neither were infected, no evidence for vertical transmission was found.

This is dedicated to all of those whose patience, wisdom, and support carried me through five of the hardest but most rewarding years of my life. To them I owe everything.

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Introduction

Introduction to the problem:

Hookworm infection is one of the leading causes of iron-deficiency and anemia in rural and tropical areas of most developing countries. It is estimated to infect over 1 billion people worldwide (one-fourth of the world's population), including 350 million women of child-bearing age (Keymer and Bundy 1989). Hookworm is reportedly the cause of 1/3 of all cases of iron-deficiency anemia worldwide (Roche and Layrisse 1966), with the total blood loss worldwide amounting to the equivalent of the exanguination of 1.5 million people per day. Hookworms are transmitted primarily through contact with soil or plants contaminated with infective larvae, which migrate through the body until they reach their final destination in the small intestine. The adult hookworms attach themselves to the intestinal mucosa, from which they remove blood and impede absorption of valuable nutrients, causing profound anemia, fatigue, and malnutrition. They can also cause local inflammation and a gastroenteritis-like picture. In particular, children and women of child-bearing age can be irreparably harmed by the protein and caloric malnutrition, and by severe iron-deficiency from the chronic blood loss. In children, the anemia and malnutrition have been shown to cause significant delays in physical, intellectual, psychomotor, and cognitive development. Prenatally, maternal infection by hookworm can seriously impede the delivery of sufficient nutrients and oxygen to the growing fetus. Human hookworm infection is easily treated with inexpensive and relatively nontoxic medications. However, unless all infected persons in the population are treated, re-exposure is imminent since community behavior

patterns often result in re-infection. Eradication of the parasite from the population is then nearly impossible. Because of the absence of natural immunity to hookworm and a high rate of infection, mass chemotherapy alone is an impractical solution and must be complemented with improved sanitation and health education.

Because infection with hookworm is usually painless and the blood loss chronic, its significant morbidity and mortality are frequently overlooked. In developing countries where other socioeconomic problems seem more immediate and visible, the global magnitude of the geohelminth problem often goes unrecognized.

Historical background:

Hookworm had historically been ignored as a serious cause of morbidity until 1880, when an epidemic of "miner's anemia" struck a group of Italian workers while they were building the Saint Gotthard railway tunnel in the Swiss Alps. Further investigation later attributed this epidemic to *A. duodenale*, one of the two species of hookworm known to commonly affect humans. By the early 1900's, hookworm infection had proved itself to be a major public health problem in the United States and Puerto Rico. Its significant morbidity caused a measurable loss of productivity and even impeded economic growth in affected areas. As a result, public health campaigns targeting hookworm were established. The Rockefeller Sanitary Commission was set up in 1909, originally to eliminate the parasite from the US and Puerto Rico. It expanded its scope to a global scale in 1913 (Ettling 1990, Keymer and Bundy 1989, Hotez 1995b). These campaigns continued until after World War II, when improved socioeconomic conditions succeeded in significantly reducing the spread of this organism in the US,

probably more so than the efforts at intervention. Hookworm is still anecdotally reported to occur in rural pockets of the south and southeastern United States, but they seem to be disappearing quickly. Worldwide, hookworm infection continues to be a major medical and public health problem, and has become a marker for inadequate sanitation and health education policy in underdeveloped countries (Pawlowski *et al.* 1991).

Much of the knowledge regarding the natural history, life cycle, and pathogenesis of human hookworm was elucidated by those funded by the Rockefeller foundations, including William Cort, AO Foster, AC Chandler, J Allen Scott, and Norman Stoll. The life cycle of *A. duodenale* was worked out in 1843 by Looss, a German government scientist working in Egypt, while that of *N. americanus* in 1902 by Stiles, a US government scientist.

Life Cycle:

Although there are many species of hookworm capable of infecting animals, the two species widely known for their propensity to infect humans are *Ancylostoma duodenale* and *Necator americanus*. Other members of the genus *Ancylostoma* - cat and dog hookworm *A. ceylanicum, A. braziliense,* and the dog hookworm *A. caninum* - have also been noted to infect humans, but only sporadically when they cause an eosinophilic enteritis syndrome. A host can be infected by either one species or by both *A. duodenale* and *N. americanus simultaneously*. In rural areas, children especially are frequently co-infected by other intestinal nematodes as well, such as *Ascaris lumbricoides* and *Trichuris trichiuris*. The two hookworms, *Ascaris*, and *Trichuris* are commonly grouped together under the classification of "geohelminths".

Hookworms are most prevalent in tropical and subtropical zones of the world, but can also be found in arid zones and on arable land if irrigation is good. *N. americanus*, known as the "New World" hookworm, occurs in warmer and wetter climate, such as South and Central America, most of Africa, southern Asia, Indonesia, parts of Australia, and some islands in the South Pacific. At one time, this species also existed in the poor rural areas of the southeastern United States (Figure 1). *A. duodenale*, known as the "Old World" hookworm, is seen primarily in the cooler and dryer areas of the tropics, such as the Mediterranean regions of Africa and Europe, northern Asia, India, and some parts of South America (Pawlowski *et al.* 1991, Hotez 1995a). They are rarely found in arid regions, which characteristically have long dry seasons.

A. duodenale are considered more virulent than *N. americanus*. They are larger worms, cause a greater loss of blood per worm, and produce a higher fecal egg count per worm (see below). *A. duodenale* have a tendency to remain in the intestine for a much shorter period of time, but their larvae have the ability to stay dormant in the body for up to 8-9 months. *A. duodenale* have several other modes of transmission in addition to its usual percutaneous route, which is *Necator*'s sole route of infection (Pawlowski *et al.* 1991, Hoagland and Schad 1978). Considering that infants and very young children are infected almost exclusively by *A. duodenale* (the more virulent of the two), the likelihood of becoming severely anemic, malnourished, and growth retarded are much greater.

	Ancylostoma duodenale	*Necator americanus*
Size, mm	8-13	7-11
Daily blood loss, ml/worm	0.20 (0.14-0.26)	0.04 (0.02-0.07)
Daily egg production/ female worm	10,000-30,000	5,000-10,000
Sex ratio, male:female	1:1	1.5:1
Adult lifespan, years	1	4-5

(Hoagland and Schad 1978)

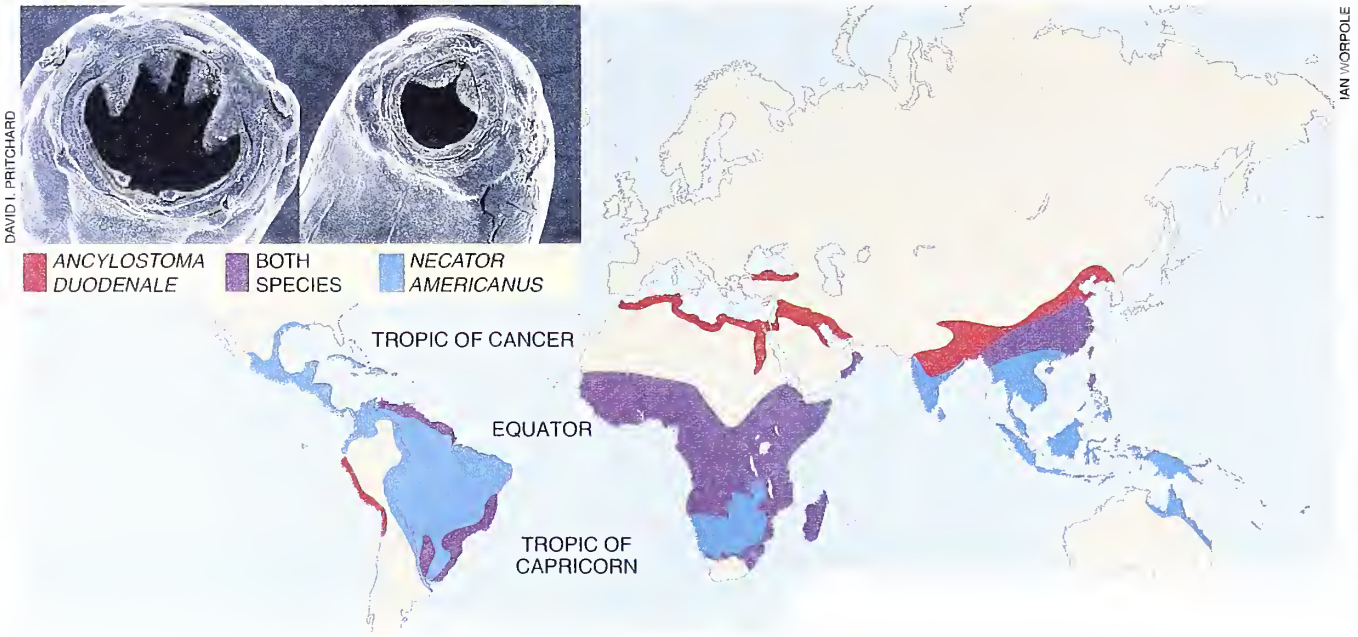


Figure 1. Distribution of human hookworm by species throughout the world. Most *A. duodenale* and *N. americanus* hookworms reside in the tropics. Red and blue colors indicate one of these species predominates in the region, but the other species may be found in small foci (Hotez and Pritchard 1995).

Adult worms (L5) are easily distinguished with a hand lens, without the need for a microscope. Females are slightly longer than males, 9-13 mm versus 5-11 mm, and have curved anterior ends. Males of both species have distinct copulatory bursa. *Necator* are much more slender and curved than *Ancylostoma*, and have a distinctive hook on their anterior ends. Microscopically, *Ancylostoma* have 2 pairs of teeth, while *Necator* uses 2 rounded cutting plates. While adult worms can be speciated easily, their eggs are not distinguishable even by microscope. After hatching, larvae of both species at or after the L3 stage can be distinguished microscopically using morphologic criteria established by the WHO. A more sophisticated, microbiological technique has recently been developed by Hawdon (Hawdon 1996) which identifies the species of either hookworm eggs or larvae using polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP).

Figure 2 represents the life cycle of human hookworms (Hotez and Pritchard 1995). Hookworms live attached to the duodenum and jejunum of the small intestine, where they feed on intestinal mucosa and blood. They mate in the intestine, then the female expels her eggs which pass with the feces. Cell division begins during passage in the intestine, and are usually at the 4- to 8- cell stage at time of defecation. In warm, moist conditions outside of the body, the eggs develop and hatch into first stage (L1) rhabditiform larvae. At 25-35°C this can occur within 24-48 hours, or longer if incubated at lower temperatures. For example, at 15°C, 90% of A. duodenale larvae and only 60% of Necator larvae have hatched by 130 hours (Hinz 1985). They fail to hatch at temperatures above 40-45°C (Pawlowski *et al.* 1991).

The free living L1 larvae are not infective, and live in and feed on the organic material in the feces or in warm moist soil polluted with feces.

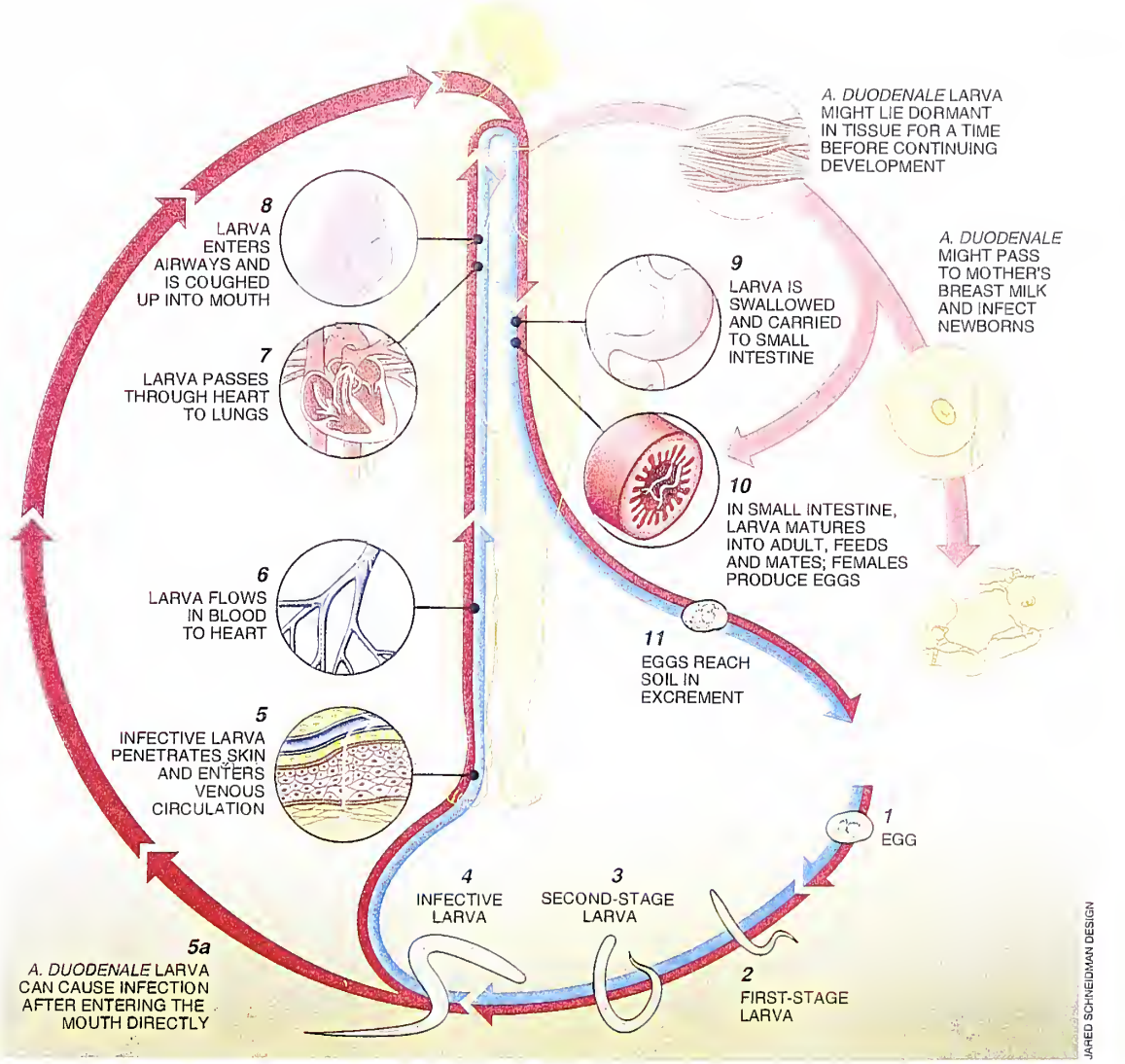


Figure 2. The life cycles of *A. duodenale* and *N. americanus*. Parts of the life cycles coincide. They "begin" with the hatching of the hookworm eggs and the development of larvae in the ground. Infective larvae generally enter the body by penetrating the skin. They move through the venous circulation, heart, lungs, and stomach before lodging in the small intestine. There they mature into adults and mate to produce fertilized eggs which pass with the feces into the ground. *A. duodenale* larvae can also establish infection in the small intestine after being orally ingested. They are also thought to take detours through the body - lying dormant in muscle tissue before reactivating to move back into the small intestine, or entering the mammary glands of pregnant women to be passed to newborns via breast milk (Hotez and Pritchard 1995).

Larvae prefer shade, moisture, loamy soil, and vegetation, and can live up to 6 weeks in this environment. Saline soils, rapidly draining soils, and waterlogged soils are all unfavorable living conditions for the larvae, and unshaded open areas provide no protection from heat and ultraviolet radiation (Hinz 1985). Within 5 days, they undergo two successive molts to become infective, highly motile, non-feeding L3 larvae, 0.5-0.7 mm in length. These larvae are barely visible to the naked eye when suspended in solution. Capable of active vertical movement, they migrate upwards using water droplets onto low vegetation and to the superficial layers of the ground where they have the best chance for contact with human skin. There is a high risk for desiccation, and most L3 larvae are able to live for only a few days unless there is some moisture and shade.

Transmission of hookworm L3 larvae usually occurs by percutaneous penetration of the skin upon contact, probably aided by the larval release of proteolytic and hydrolytic enzymes. They enter the venous circulation, passively migrate through the heart, and worm their way into the alveoli of the lungs. They move up the bronchioles, bronchi, and trachea, until they are subsequently swallowed at the pharynx. They are then carried to the duodenum and jejunum, where they attach to the mucosa, mature into adults, mate, and the female expels her eggs. Migration of hookworm larvae from the time they enter the body through the blood circulation and lungs into the intestine requires 3-5 days. About 6-8 weeks after skin penetration, the hookworms reach maturity and are able to produce eggs.

The transmission of *Necator* occurs strictly by the percutaneous route. *A. duodenale* larvae infect percutaneously, but can also cause infection via ingestion of food or water contaminated with larvae (Hoagland and Schad 1978). In this case, the larvae move directly to the intestine, bypassing the

circulation and lungs. Unlike *Necator*, *A. duodenale* larvae have also displayed an ability to arrest their growth and to remain dormant in various body tissues (muscles, intestines) for up to 200 days (Schad *et al.* 1973) before reactivating and resuming the maturation process. It had been postulated that, if *A. duodenale* larvae were able to penetrate various tissues, they may also be transmitted transplacentally and/or via breastfeeding. Evidence for transplacental transmission has not been found, though transmammary transmission still remains a possibility. Both the issues of arrested development and transmammary transmission have very important epidemiological implications, and will be discussed in detail below.

Arrested development (hypobiosis):

A. duodenale larvae have been found to have the ability to arrest their development in the host (hypobiosis), and maintain this state until external conditions (such as the soil or moisture conditions) are optimal for shedding eggs. Reactivation is likely to occur with temporal/seasonal periodicity, i.e. immediately preceding the monsoon season, to ensure peak egg deposition in warm, moist soil. Stool studies performed by Schad *et al.* (1973) in West Bengal, India found a marked increase in egg production preceding (not following) monsoon season. Considering the natural history of hookworm maturation, the 6-8 week period required for maturation would place maximum infection rates during the hot, dry months before the monsoon season. However, these conditions would be inhospitable for large numbers of hookworm larvae, and soil samples taken at this time were negative for larvae. Heavy transmission could not have occurred immediately before the increase in egg production seen early in monsoon season. Rather, infection most likely occurred during the previous year's monsoon season, and the

larvae remained dormant in the body of the host until conditions for optimal egg-laying were imminent. Also, the study reported that conversion from negative to positive infection occurred late in the rainy season. If typical maturation was to commence after conversion, a rise in egg count would be seen post-monsoon. This is not seen.

Other approaches have been taken to prove the existence of arrested development. Self-induced *A. duodenale* infections were followed by carefully monitoring of the stool to detect the first appearance of the eggs (Schad *et al.* 1973, Nawalinski and Schad 1974). Nawalinski and Schad first showed an eosinophilia and leukocytosis starting at 33 weeks post-infection, then ova appearing in the stool at 40 weeks post-infection. Likewise, Schad *et al.* (1973) showed ova to first appear in the stool at 9 months post-infection. It is possible that the larvae were not truly dormant in the body, but just developing more slowly. However, the distinct rise in eosinophilia and leukocytosis just 7 weeks before patency of infection more likely suggests a reactivation phenomenon.

A. duodenale seems to be the only human hookworm capable of hypobiosis. Seasonal variation in hookworm egg counts has been noted only in the presence of *A. duodenale* or mixed-species infections. Populations infected exclusively with *Necator* have not shown this seasonal variation (Roche and Layrisse 1966, Schad *et al.* 1973), implying that only *A. duodenale* had the capacity for arrested development. These results are supported by a report from China about a group of people recently infected with hookworms who were given anthelminthics, and subsequently expelled immature forms of both *A. duodenale* and *Necator*. With no chance of re-exposure, they were reported to have been feeling well until 6-7 months post-treatment, when some members began having symptoms of hookworm infection again. Upon

re-treatment more hookworm, exclusively *A. duodenale* of both mature and immature forms, were expelled (Schad 1990). Only arrested *A. duodenale* larvae were able to survive initial chemotherapy to be reactivated at a later date.

There is also a major question as to *where* these arrested larvae may be found in the body. One possibility is that most of them remain in the gastrointestinal tract. Reports that arrested *A. duodenale* larvae have been found in skeletal muscle and other somatic tissues of humans have been primarily anecdotal, though Little *et al.* (1983) found an *Ancylostoma* L3 larvae (species unknown) in a human muscle biopsy specimen after a severe case of cutaneous larval migrans. It is a well-documented fact in dogs with *A. caninum* infections that *A. caninum* (a closely related species) larvae have been found in their musculature (Schad 1990). Along the same lines, experimental infection of calves, pigs, lambs, rabbits, and chickens with *A. duodenale* larvae have shown larvae hibernating in the somatic musculature of all these animals except chickens (Schad 1990). Not only has *A. duodenale* proven a capacity for arrested growth, but meat-borne transmission now becomes another hypothetical mode of transmission (Gilles 1985).

Arrested development implies that, at reactivation, there must be mobilization of the *Ancylostoma* larvae from the muscles into other tissues, such as the lungs and maybe into the circulation. The possibility of larvae "hibernating" or mobilizing in body tissues outside of the intestine could be the key to explaining why *A. caninum* infective larvae have been found in unlikely places, such as the colostrum of female *A. caninum*-infected dogs (Stone and Giradeau 1968).

Vertical transmission

The above finding becomes extremely important because it suggests another possible route of transmission for *A. duodenale* - vertical transmission from human mother to infant either pre- or postnatally. Vertical transmission, either transplacentally or lactogenically, is a well-known phenomenon in other helminths and the zoonotic *Ancylostoma* species. Prenatal transmission of *Toxocara canis* is well-documented in dogs, and has recently been shown to be transmitted lactationally as well (though with significantly less frequency) (Burke and Roberson 1985, Stone and Giradeau 1968). *Strongyloides stercoralis* has also been known to transmit their larvae lactationally, and *Strongyloides* L3 larvae have even been found in human breast milk samples from heavily infected mothers (Brown and Giradeau 1977). And in the above-mentioned *A. caninum*-infected female dogs, hookworm larvae were actually isolated from canine breastmilk, and lactogenic transfer of larvae was the cause of fulminant *A. caninum* infections in the pups (Stone and Giradeau 1968). No transplacental transmission of *Ancylostoma* has yet been demonstrated in animals nor in humans (Burke and Roberson 1985), but its possibility cannot yet be excluded.

Typical percutaneous transmission is improbable in cases of very early infantile ancylostomiasis because 1) environmental exposure is usually minimal during the first few months of life, and 2) percutaneous infection requires 6-8 weeks (average 53 days) before infection can be detected in stool samples, and infants as young as 20 days old have been reported to be infected (Yu and Shen 1990, Yu *et al.* 1995, Wang 1988). From examples of *A. caninum* infection in dogs, *A. duodenale* infective larvae could hypothetically be found in human colostrum (and maybe early breastmilk), which is only produced during the first few days postpartum. This means that the window of

infectivity from mother to her newborn is very short. No one has yet been able to isolate hookworm larvae from human colostrum or breastmilk (Brown and Giradeau 1978, Nwosu 1981). An ethical dilemma surrounds the extraction of colostrum, since depriving newborns of this early milk may deprive them of the valuable immunoglobulins and other protective factors contained in it. The next most direct way of searching for evidence of vertical transmission in humans is to study reported cases of infantile ancylostomiasis. In a study by Brown and Giradeau (1977) in Zaire, although no hookworm larvae were found in breastmilk samples of lactating human mothers, 8% of the infants (4 of 76) under the age of 200 days were already positive for hookworm eggs in the stool (one infant as young as 37 days). However, the mothers were not tested for hookworm infections, and the possibility for environmental exposure was not ruled out. Studies in China have identified at least 500 infants, many as young as 12-20 days old, with *A. duodenale* infections and clinical symptoms (Yu *et al.* 1994, Yu *et al.* 1995), though many had probably been exposed by sand-filled diapers. However, these studies also report individual cases of young infants who were hookworm-infected yet had no environmental means of acquiring the infection. If transmammary transmission (rather than environmental exposure or sand-stuffed diapers) *is* actually the cause of most cases of infantile hookworm infections, this would explain the large predominance of *A. duodenale* (versus *N. americanus*) infection in infants seen in China (Yu *et al.* 1995) , West Bengal (Nawalinski *et al.* 1978), and all other studies. In areas where only *N. americanus* occurs, evidence for vertical transmission has been sought but never found (Schad 1990).

In the case of postnatal transmission, the resumption of development after the period of larval arrest probably occurs at the onset of pregnancy or at

parturition (Hotez 1995b), and is thought to be caused in part by hormonal changes. Investigators are looking particularly at the involvement of steroid or prolactin receptors in the reactiviation of larvae. Evidence thus far has been inconclusive, and is mostly hypothetical at this point.

Clinical manifestations:

During the route from invasion to final implantation in the gut, the invasive L3 larvae can cause a string of temporary clinical pathologies. At the site of percutaneous invasion (usually between the toes, feet, legs, and buttocks), there may be a stinging sensation followed by irritation, erythema, and papulovesicular eruptions - commonly known as "ground itch". It is often more prominent with infection by zoonotic hookworm species (e.g. *A. braziliense*) or in those who are visitors to the area. Zoonotic species can also cause a condition called cutaneous larva migrans. Because the larvae are incapable of penetrating through dermal layers of the skin, they migrate horizontally through the epidermis, leaving a discernible trail of inflammation and erythema.

For human hookworm L3 larvae, migration through the tissues into the circulation causes little pathology, though small hemorrhages and an eosinophilic infiltrate may occur upon penetration of the alveoli through the alveolar capillaries. As they pass through the respiratory system, they may irritate the airways to cause coughing. Once lodged in the gastrointestinal tract, they attach onto the mucosa and, aided by a secreted anticoagulant (Hotez and Pritchard 1995, Hotez 1995b), feed on mucosa and blood. The effects of the anticoagulant remain long after the hookworm disengages, and the bleeding may continue. They often cause chronic epigastric pain, indigestion symptoms, anorexia, and diarrhea. Cases of infantile

ancylostomiasis in China describe pictures of severe melena, tarry stools, and anorexia (Yu *et al.* 1995, Wang 1988). Pica may also be observed, since it is known to relieve some of the gastrointestinal symptoms. Marked eosinophilia in the blood can be seen, and eosinophilic gastroenteritis has been noted (Prociv and Croese 1990).

More concerning than the acute symptomatology is the long-term consequence of hookworm infection. Most of the symptoms and signs of infection result from a loss of blood, nutrients, and serum proteins via the GI tract. Chronic anemia results in feelings of weakness, fatigue, decreased stamina, and exertional dyspnea. Work productivity may be compromised (Gilles 1985). Palpitations, dizziness, chest and extremity pain are also common complaints. Those infected chronically with hookworm may have pallor of the skin, conjunctiva, and tongue, or even a distinctive yellow-greenish tinting of the skin known as "chlorosis". Increased pulse pressure and peripheral vasodilatation may be present. Koilonychia and angular stomatitis may result from the iron-deficiency. Very severe anemia may cause a congestive heart failure-type picture. The hypoproteinemia manifests itself as severe upper and lower extremity edema, ascites, and a kwashiakor-like picture in infants and children (Hotez 1989, Variyam and Banwell 1982).

A correlation has been shown between fecal egg counts (hence, worm burden) and actual degree of anemia (Variyam and Banwell 1982, Roche and Layrisse 1966, Gilles 1985). Iron-deficiency is already a major problem of many underdeveloped countries due to low dietary intake. Especially in Central and Southeast Asia, rice is the staple food, little meat is eaten, dietary ascorbic acid is low, and there is a high intake of tannates (in tea) which impairs iron absorption (Pawlowski *et al.* 1991). These problems usually do not exist in

developed countries, where iron-fortified foods and proper nutrition are complemented by a higher level of sanitation.

Children and women of child-bearing age are especially vulnerable since their physiological needs for iron are high. As stated earlier, the more virulent *A. duodenale* is the predominant hookworm found in infants. In the younger ages, infection causes apathy, listlessness, growth retardation, slower cognitive development, and failure to thrive due to anemia and nutritional deficiencies. Iron is essential for the functioning of dopaminergic neurons and for the synthesis of certain neurotransmitters (Scrimshaw 1991). Others have shown that severe iron deficiency leads to reduced iron-containing enzymes such as aldehyde oxidase and monoamine oxidase, enzymes which are necessary for the breakdown of the neurotransmitters serotonin and norepinephrine. Increased serotonin levels can cause decreased attentiveness, while an excess of norepinephrine produces irritability. Lower intelligence test scores and scholastic performance can be seen, as well as developmental slowing. The reversibility of these deficits depends on the timing of the delays, and may be irreversible.

Physical growth impairment can also be severe, and occurs primarily in heavier hookworm infections. This delay is most apparent during adolescence, a time when there is supposed to be the greatest rate of growth. It is encouraging that physical growth delays are at least partially reversible if nutrition is promptly corrected and the child is given anthelminthics and iron supplementation. In longitudinal studies of Kenyan school children (Stephenson *et al.* 1989a), primary schoolchildren infected with hookworm, *Ascaris*, and/or *Trichuris* were treated with single-dose albendazole. Stool exams and anthropomorphic measurements performed 6 months later showed significant differences in infection intensity and in the growth rates

between those who were treated with anthelminthics versus those who received placebos. The growth parameters measured were weight, %weight for age, %height for age, %weight for height, %arm circumference for age, and skinfold thickness. Though many children were mildly re-infected by the study's end, even decreased infection intensities were significant predictors of improved (increased) growth parameters - 6 out of 6 when hookworms levels were decreased, and 4 out of 6 when *Ascaris* and *Trichuris* levels were decreased. There were actually significant decreases in 5 of the 6 growth parameters for those in the placebo group, showing almost a "growth regression". The study by Stephenson *et al.* is important because it also suggested that major decreases in parasite load were sufficient to promote growth, and that maintenance of a parasite-free state was not necessary to see significant improvement. Modified studies performed by the same investigators using different chemotherapies and different indices of improvement have shown similar results (Stephenson *et al.* 1989a, 1989b)

In pregnant women, hookworm infection can cause severe iron-deficiency anemia, intrauterine growth retardation, and compromise to the fetus.

Mortality for hookworm are generally low, with an estimated 50,000-60,000 deaths a year attributed directly to hookworm (Walsh and Warren 1979). Many of these deaths are of young children and infants. Infant mortality has been estimated to run as high as 4.2-12% of all cases of infantile ancylostomiasis in certain endemic areas (Yu *et al.* 1995). The morbidity is also higher in infants and young children than in adults. Hookworm is estimated to cause an astounding 1.5 million hospital visits a year from the large number of complications described earlier (Pawlowski *et al.* 1990), of which infants comprise 7.4-29% (Yu *et al.* 1995).

Diagnosis and treatment:

The presence of hookworm can be easily determined by identifying their characteristic eggs in a stool sample. Many quantitative techniques are available, including the Kato-Katz stool smear, the Beaver's direct egg-count technique, the Stoll's dilution egg-count technique, and the McMaster's technique. However, the species of hookworm cannot be identified by looking at their eggs alone. Examination of either L3 larvae or adult worms is necessary to distinguish between *A. duodenale* or *N. americanus*. For the purpose of species identification, the larvae of the human hookworms have traditionally been distinguished from one another via well-established morphological characteristics. This method is very convenient when doing research in the field. The is also a new molecular method of species assignment developed by John Hawdon in the Hotez lab which uses a PCR-RFLP technique (Hawdon 1996).

Chemotherapy for hookworms involves the use of the anthelminthics mebendazole, albendazole, levamisole, or pyrantel pamoate. They are also all effective against *Ascaris*. *Necator* is reportedly less susceptible than *A. duodenale* to treatment with levamisole and pyrantel. Annual or bi-annual treatment with anthelminthics is recommended for large-scale hookworm control programs in endemic areas, especially if transmission is seasonal. Re-infection with hookworms invariably occurs in endemic areas, so that the exclusive use of anthelminthic medications is not a sufficient control method. Chemotherapy must be coupled with improved sanitation efforts and health education to have any hope of controlling the problem long-term. In addition, these medications historically have not been well-absorbed by the GI tract (since they are designed to stay within the intestine to treat the worms), so therefore any arrested *A. duodenale* larvae may not be treated.

However, there has been recent evidence in *A. caninum*-infected mice that metabolites of albendazole may be active in eliminating the larvae from the muscle tissue (Xiao *et al* 1994).

Epidemiology and population biology:

Climate sets the general limits of distribution of hookworm infection, but local prevalence and intensity are largely determined by human activities (Pawlowski *et al.* 1991).

As stated earlier, an estimated 1 billion people are infected with hookworm worldwide: 685 million in Asia and Southeast Asia, 132 million in Africa, and 104 million in Central and South America (Pawlowski *et al.* 1991). In China alone, hookworm is estimated to infect 194 million inhabitants, (17% of the population) (Xu *et al.* 1995), with *N. americanus* more dominant in southern China (warmer, wetter climate) and *A. duodenale* in the north (cooler, drier climate). At present, the distribution of *A. duodenale* and *N. americanus* is unknown in the area of our studies along the Thai-Myanmar border.

The pattern of hookworm transmission and its overdispersion is determined by two key factors: the degree of exposure to infection, and the degree of resistance (immunocompetence) of the host. The distinct effects of these individual factors is remains unclear and will be discussed in later paragraphs.

In terms of exposure to hookworm disease, there are several factors commonly observed in rural and developing areas that ensure transmission and propagation of hookworm at the community level. These "endemic" factors include the general lack of sanitation in a community, the lack of designated sites for defecation (hence, "indiscriminate" defecation), and the

use of uncomposted human feces (nightsoil) as fertilizer in small-scale farming. These are integrated behaviors of the community and its culture. "Epidemic/focal" outbreaks usually occur among miners and tunnel workers, and day laborers under conditions that are favorable for transmission.

In very underdeveloped areas, there often is no designated bathroom or outhouse, so inhabitants of the community defecate in the woods, under bushes, or at the edge of agricultural fields. Conditions here are ripe for hookworm larval development since they are often shady and moist. Habitual travel to these heavily polluted areas by many members of the community will lead to rapid infection, and re-infection, by hookworm.

The risk of infection is even higher if proper footwear is not worn. Children are universally known to play while barefoot. In cultures which require frequent removal of shoes (to step in and out of houses) and in tropical countries, footwear usually consists of open-toed rubber flip-flops or thongs, which give little resistance to larval infection.

Hookworm is frequently seen as an occupational disease of farming communities (Gilles 1985). Agricultural activities play a major role in the transmission of hookworm. Certain crops, such as mulberry leaves for silkworms, coffee beans, sugar cane, and tea, grow in environments which favor the cultivation of hookworm larvae - shady, loamy soils with irrigation. Long days in the field and large work forces lead to frequent defecation in the fields. Coupled with the fact that agricultural workers often work barefooted, the spread of hookworm is inevitable. The situation is further exacerbated by the widespread use of nightsoil (human excrement) as a fertilizer, as is very common in China (Wang 1988, Xu *et al.* 1995, Yu and Shen 1990). In fact, vegetable growers and farmers showed the highest

prevalence rate for hookworm than any other profession (students, fishermen, herdsmen, teachers) in China (Xu *et al.* 1995, Yu *et al.* 1994).

Along with the possibility of transmammary transmission, infants are exposed to additional modes of hookworm infection. Infants are often set down on the ground or onto sandbags and wheat-stem bundles while their parents are working in the fields. And in many cultures, infants are also diapered with cloth sacks stuffed with sand and dirt, bringing them into close contact with contaminated soil (Yu *et al.* 1995, Wang 1988). Wang (1988) estimates that approximately 46% of infantile hookworm infections in China can be attributed to sand-stuffed diapers.

The rate of transmission can also be affected by other factors contributing to exposure (usually behavior patterns), such as cooking and eating rituals, bathing practices, clothes and dish washing locations, floor types in houses and outhouses.

A community may not be homogenously infected with hookworm. In communities where overall prevalence of hookworm is low, there may be focal areas of high infection levels. Likewise, in communities where overall prevalence of hookworm is high, there may be small localities where infection levels are lower.

The intensity of infection also varies widely between individuals and groups in a given community, with a small percentage of the people harboring a disproportionately large fraction of the total hookworm load. Epidemiologically, the distribution of hookworm in endemic areas worldwide is overdispersed, with more than 65% of the worm population aggregated into less than 15% of the host population. A nationwide study of China involving nearly 1.5 million subjects (Xu *et al.* 1995) showed that 75.4% of the subjects were identified as having light infections (<400 eggs per gram

feces), and that 70% of the total parasite load was harbored by only 15-30% of the study group.

In all studies of hookworm, the pattern of overdispersion resembles a negative binomial distribution (Figure 3) (Bundy 1990), where the probability of infection generally varies inversely with the intensity of infection. In some but not all of these frequency distributions, there is also a small hump at the tail of the curve, representing a cluster of people with especially high infection intensities. In looking at the overdispersed pattern of hookworm distribution, in which a small fraction of the population harbors a large fraction of the worms, the question of predisposition/vulnerability naturally follows. Is it possible that this small fraction of people is, for some reason, biologically or *inherently* more susceptible to frequent or heavier infections, or do socioeconomic and transmission-linked factors explain the variability entirely?

"Predisposition" is a term used to describe the extent to which these infection characteristics are determined by inherent host features (Keymer and Pagel 1990), and may result from biological, ecological, genetic, social or behavioral factors acting alone or in combination (Schad and Anderson 1985). Host factors such as age, sex, and immune status may all contribute to the variability observed between hosts (Keymer and Pagel 1990). Measuring predisposition is difficult, and has been done indirectly with socioeconomic, behavioral, and nutritional studies. Direct evidence has only come using longitudinal intervention studies, in which the rate of re-infection is observed for months and years after chemotherapy and compared to pre-medication levels. In a study by Schad and Anderson (Schad and Anderson 1985), pre- and post-treatment levels of hookworm infection were measured in an endemic area of West Bengal, India in 2-month intervals for 16-18

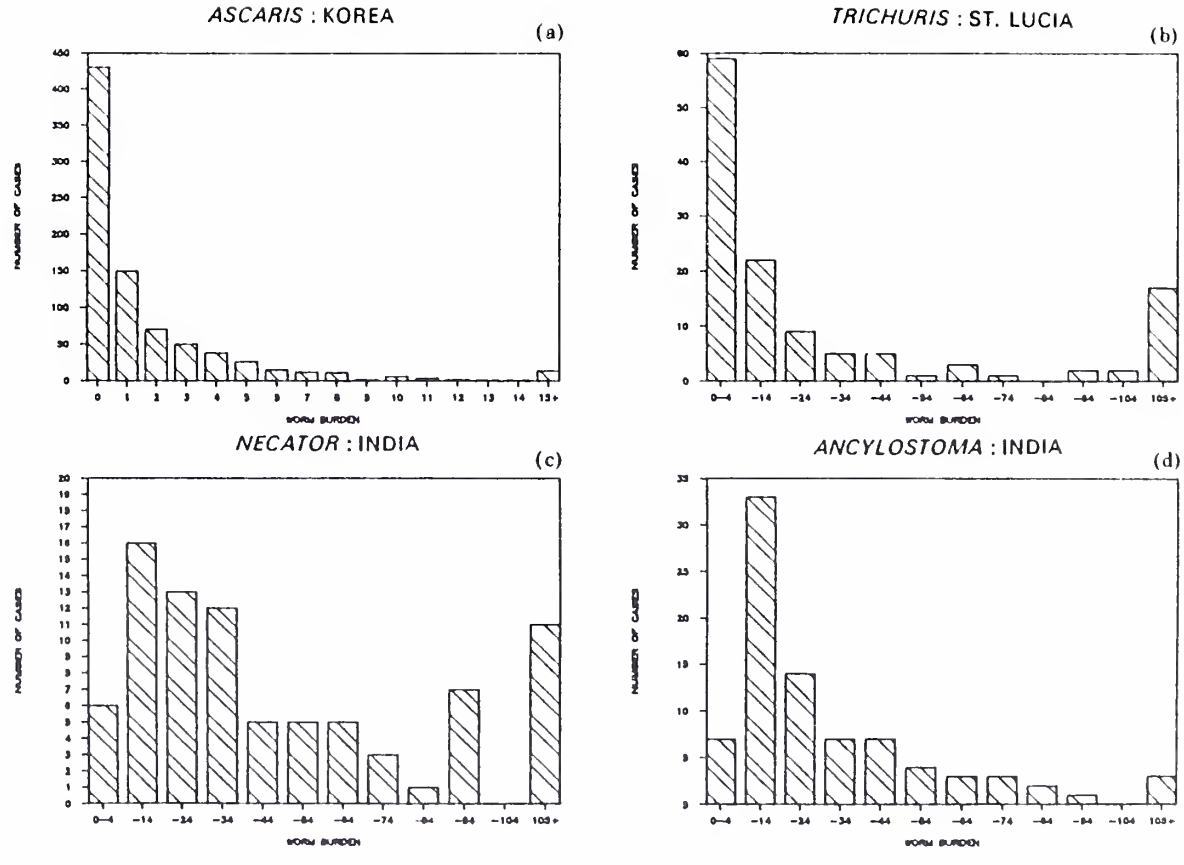


Figure 3. Frequency distributions of the major geohelminths of humans - *A. duodenale, N. americanus, Ascaris, and Trichuris.* All distributions are markedly overdispersed, and were estimated from worm burdens obtained by anthelminthic expulsion (Bundy 1990).

months. Not surprisingly, the frequency distributions of both species of hookworm were highly aggregated, with greater than 60% of the total hookworm population held by less than 10% of the studied population. The study also showed statistically significant positive associations between pre- and post-treatment levels of infection in all rounds of reexamination (Kendall tau correlation coefficient=0.67, $P<0.001$).

This study was included in a review by Bundy of 12 studies on predisposition (Bundy 1990), all comparing pre- and post-treatment helminth (hookworm, ascariasis, trichuriasis, enterobius, schistosomiasis) infection levels in individuals. Eleven of the 12 studies showed weak but statistically significant evidence for predisposition - those with high levels of infection initially tended to have relatively high levels post-treatment, and tended to become re-infected more quickly.

This method of looking at pre- and post-treatment levels to determine degree of predisposition is tricky since it can be biased by the following factors. Infection intensity for all of the helminths is age-dependent, and predisposition is easier to detect in age groups in which infection intensity is highest. Another confounding factor is the method used to measure intensity of infection - eggs per gram versus worm expulsion. Phenomenon such as density-dependent fecundity (discussed later) will make the former method a less accurate way to determine parasite load. Lastly, the most accurate way of determining predisposition is by measuring post-treatment infection levels after the study population has reached an equilibrium state, which can take months to years. The time intervals between measuring the pre- and post-treatment infection levels varied considerably among the 12 studies.

The age-dependence of predisposition was also studied by analyzing the extent of predisposition within separate age classes, ideally after correcting for age-related trends. In all 12 studies, the level of significance within age classes was generally less than that yielded by the overall sample (Bundy 1990). Unlike the overall sample, where 11 of 12 studies showed evidence for predisposition, only 19 of 30 tests within age classes gave statistically significant positive associations. Predisposition to schistosomiasis seemed to be a childhood phenomenon, but firm conclusions about the age-relatedness of predisposition could not be drawn about the other helminths (Bundy 1990). Bundy also acknowledges, however, that the results of these re-infection studies within age classes were highly variable, and may be confounded by the facts that the rate of re-infection in itself is age-dependent (i.e. children may reach an equilibrium state more rapidly than adults) (Bundy 1990) and that infection intensity is also age-dependent (see below). These factors would make predisposition easier to detect in children than adults.

Studies on re-infection and predisposition are very valuable. Not only do they give insight into a biological basis for variations in susceptibility to infection, but on a more practical level they also identify those who are repeatedly likely to have higher hookworm loads. These people theoretically form groups which can be targeted for regular chemotherapy, and this information is helpful for planning control programs (Anderson and May 1985, Anderson and Medley 1985). Studies in laboratory animals and human volunteers have shown that males inherently may be more susceptible than females to hookworm infection, and Caucasians more so than non-Caucasians (Schad and Anderson 1985), though in China females seem to have a higher hookworm prevalence than males. There may be an element of biological influence on predisposition, and laboratory models of infection

to date have suggested a genetic basis for resistance and susceptibility (Schad and Anderson 1985).

Age-relatedness of prevalence of hookworm infection:

Although the age-relatedness of "predisposition" is entirely unclear, there are other characteristics of hookworm infection that are definitively age-related. The host is usually infected during childhood, then repeatedly re-infected throughout life. Mature hookworms survive between 1 and 5 years in the gut, depending on the species. Age-prevalence curves for hookworm have conflicted in their reputed age of peak prevalence. Most reported curves show a rise in prevalence throughout childhood until early adulthood, when prevalence plateaus. Other curves have shown peak prevalences in early or mid-adolescence before plateauing. Still other curves show a clear peak in late childhood (Figure 4) (Bundy 1990). This is different from the other helminths like *Ascaris* and *Trichuris*, which show a consistent peak during early childhood of both prevalence and intensity of infection from study to study. One explanation for the inconsistent curves found in hookworm studies may be the fact that *A. duodenale* and *N. americanus* have traditionally been grouped together during epidemiological analysis without much regard for their differences. As explained earlier, *A. duodenale* and *N. americanus* have many characteristics that distinguish them from one another, including virulence factor, geography, and possibly the age groups they tend to infect.

Most studies show that prevalence tends to remain constant throughout adulthood, though some would argue that it actually decreases slightly with age.

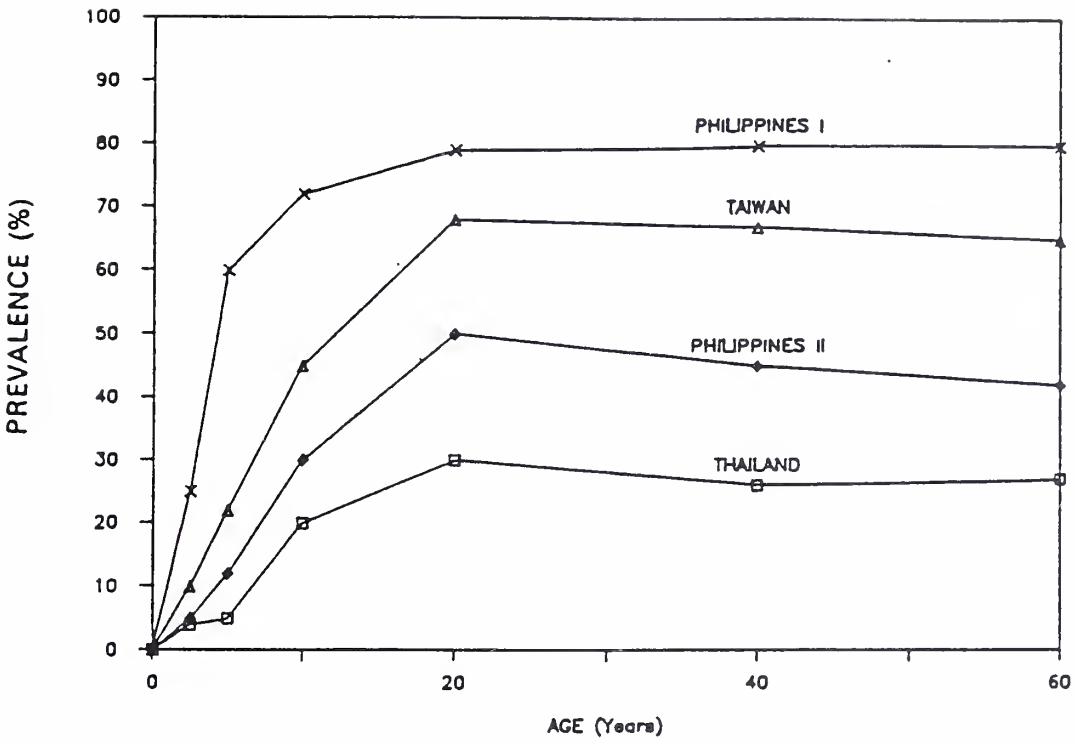


Figure 4. Age-prevalence profiles for mixed hookworm infections. Each line represents a different population and a different geographical location. Each population reaches its maximum prevalence at different ages. (Bundy 1990).

Age-relatedness of intensity of hookworm infection:

Studies of age versus intensity of hookworm infection show similar patterns as prevalence, that the worm burden carried by the host rises almost linearly throughout childhood until there is a peak intensity at approximately 20-25 years old. Into adulthood, the intensity of infection reaches a plateau and may even fall slightly. Intuitively this makes sense. An increase or decrease in one (prevalence or intensity) would inevitable lead to a similar increase or decrease in the other, since they both represent the level of hookworm infection found in the community.

Like the prevalence, many studies performed on the age-intensity relationship have given conflicting results concerning the intensity of hookworm infection as hosts aged. One viewpoint is that intensity plateaus or continues to rise slowly with increasing age. Others feel that the intensity of infection peaks then starts to fall with age, causing a convex-looking graph (Figure 5). Their explanation for the convexity in some of these graphs may be that repeated re-infection may induce an immunological response and acquired immunity by the host over time: at younger ages the level of infection in a host is determined by exposure, while at older ages the level of infection is determined by both exposure and the strength of the host's immunological response to counter the infection. Another explanation is that the hosts' immune system may simply evolve/mature independently with age, without the induction of immunity via infection.

The argument over "exposure versus immunity" as the major determinant of level of hookworm infection in certain age groups (older age groups in particular) is not resolvable at this time. The major factor governing worm burdens is the level of exposure of the host to the parasite, though immunology and predisposition seem to affect infection intensity to

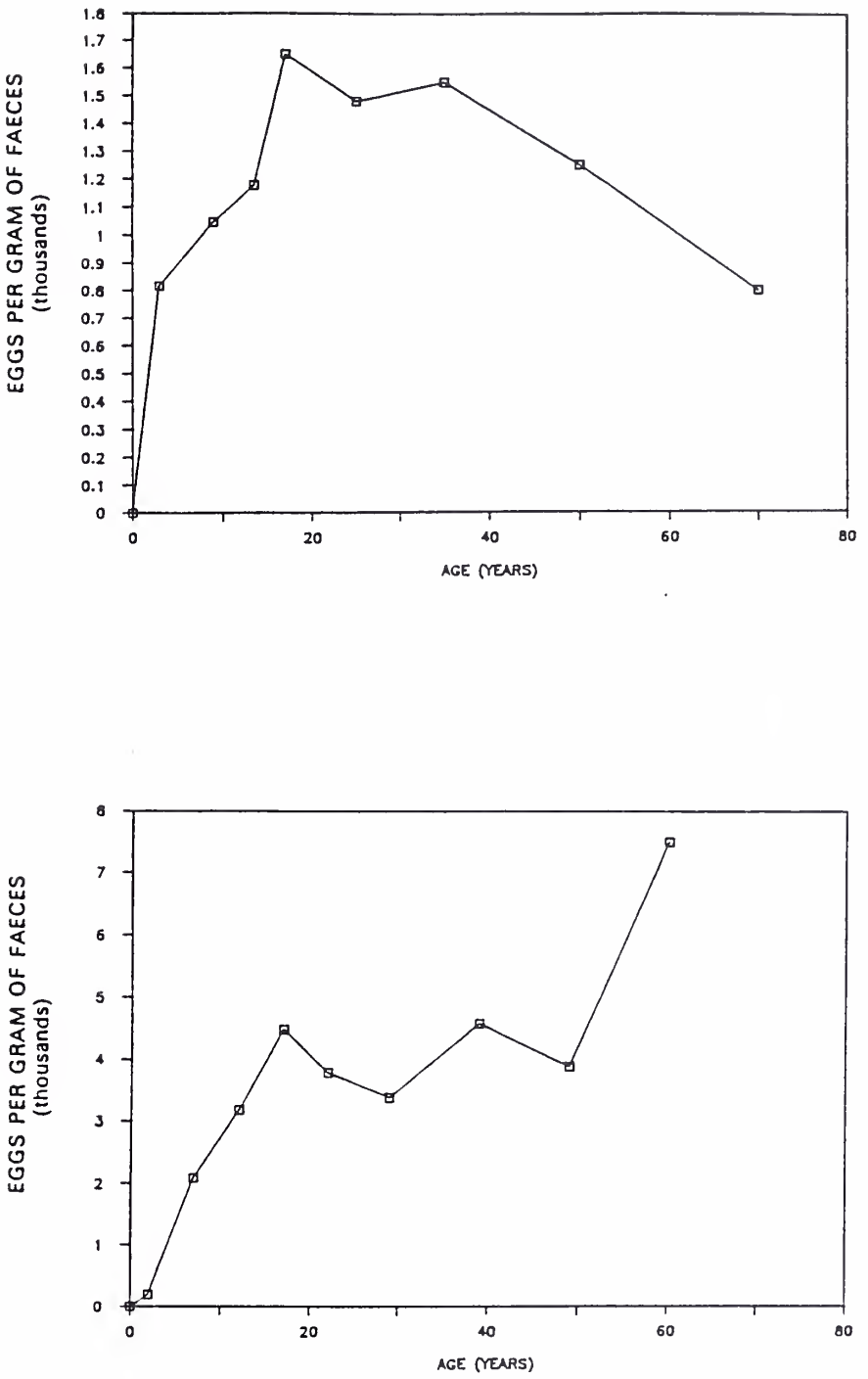


Figure 5. Two extreme examples of age-intensity profiles of mixed hookworm infections (as estimated by measuring eggs per gram feces). Each graph represents a different population and a different geographical location. Together they demonstration the wide degree of variability in which hookworm can infect a population (Bundy 1990).

some degree (Warren 1988). It is difficult to tease out the independent effects of these factors, since quantitative studies of exposure are very difficult to construct. The degree to which the host's immunology can modulate infection intensity is another major question. Evidence for acquired immunity include a study by Anderson and May (1985), which found that the rate of decline of infection intensity was more rapid in areas of high transmission than in equivalent areas of low transmission for both schistosomes and hookworm (Figure 6) (Anderson and May 1985). It has also been found in studies of immigrants to schistosome-endemic areas that intensity of infection is determined by the duration of residence in the endemic area, rather than by actual age (Kloetzel and daSilva 1967). This result will be especially important for our studies in Thailand, in which refugee (transplanted) populations make up our study groups.

Density-dependent fecundity

Lastly, a discussion of density-dependent fecundity is highly warranted in studies attempting to quantify levels of infection or measure infection intensities. In all hookworm studies, the intensity of infection is measured by either worm expulsion after treatment with an anthelminthic, or by measuring the number of eggs per gram of feces. The former requires collecting all of the stool produced by a subject after an anthelminthic is given, then washing and sifting through the stool to isolate the worms in it. The latter is frequently used since it is easier and faster to perform, and requires only a small stool sample. However, it is a much less accurate way of determining actual infection intensity for 3 major reasons. First, the number of eggs produced per female worm decreases rapidly and non-linearly as the density of worms in the gut increases (Krupp 1961, Anderson and Schad 1985).

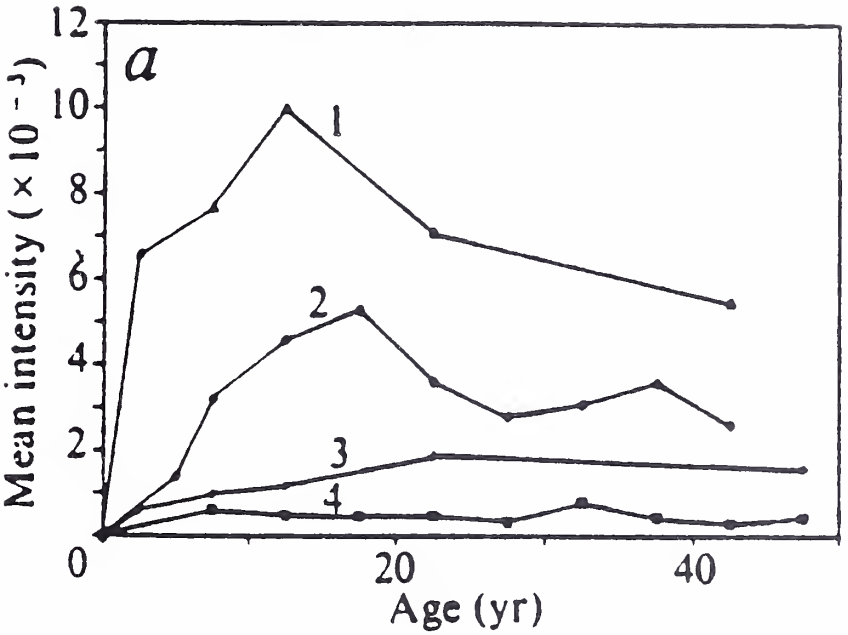


Figure 6. Mixed hookworm infection different communities with varying intensities of transmission. The degree to which intensity declines in the older people seems to be positively associated with the intensity of transmission (Anderson and May 1985).

This phenomenon, called density-dependent fecundity, starts at relatively low worm burdens - burdens at which the highest fraction of infected people carry. At higher levels of infection, the hookworm load is easily underestimated since egg production is increasingly depressed. Secondly, it is impossible to distinguish whether the infection is *A. duodenale*, *N. americanus*, or both by microscopic examination of the eggs. The net rates of egg production in *A. duodenale* and *N. americanus* are different because the density-dependent effect is more severe with *N. americanus* than *A. duodenale* (Anderson and Schad 1985). Especially in mixed infections, estimating the worm burden would be very difficult. Lastly, the day to day variance in the number of eggs produced is very wide, so multiple stool samples must be analyzed if an accurate estimate of worm burden is to be obtained (Anderson and Schad 1985).

The biological cause for density-dependent fecundity is unknown. Krupp was also able to show that smaller percentages of larvae would develop into fully mature adult worms as the host became increasingly colonized.

Polyparasitism:

In places of the world where hookworm is a major endemic parasitic problem, hosts are likely to be co-infected with *Ascaris lumbricoides* and/or *Trichuris trichiura*. The 3 parasites (commonly referred to as "the Holy Trinity") often occur together in the same geographical areas since they share similar transmission conditions. They often share hosts as well, especially in children of developing countries where protein-energy malnutrition is common, sanitation is poor, and health education is sparse. In the Chinese parasite survey mentioned previously (Xu *et al.* 1995, Yu *et al.* 1994), 17.2% of

the subjects were infected with hookworm, 18.8% with *Trichuris*, and 47.0% with *Ascaris* infections. When extrapolating these results to the entire population, the study estimated that 194 million Chinese would have hookworm infections, 212 million trichuriasis, and 531 million ascariasis, with a total number of infected people (including double and triple infections) to be 646 million. Different regions of the world also differ in the proportion of the parasites found. Compared with the distribution in China, the studies of Kenyan schoolchildren (Stephenson *et al.* 1989a) showed 87% with hookworm infections, 97% with trichuriasis, and 49% with ascariasis - the multi-parasitic infection is obvious.

There are many differences between the epidemiological patterns shown by hookworm and those shown by *Ascaris* and *Trichuris*. *Ascaris* and *Trichuris* show a very strong age-preference for young children, with prevalence of infection consistently peaking at the age of 5-10 years, then declining through adolescence and adulthood (Figure 7) (Bundy 1990, Xu *et al.* 1995, Yu *et al.* 1994). In contrast, prevalence curves for hookworm have been inconsistent, but have generally shown prevalence to peak sometime in adolescence or adulthood. Similarly, while hookworm was found to infect vegetable growers and farmers in higher proportion than other professions (teachers, fishermen, students, herdsmen) in the China study, the highest prevalence of *Ascaris* was found among students and schoolchildren (Yu *et al.* 1994, Xu *et al.* 1995).

Like the prevalence of infection, the graphs of intensity of infection show a similar age-dependence. Those with the heaviest *Ascaris* and *Trichuris* infections fell into younger age groups (0-14 years old), while the heaviest hookworm infections were carried by those greater than 20 years old.

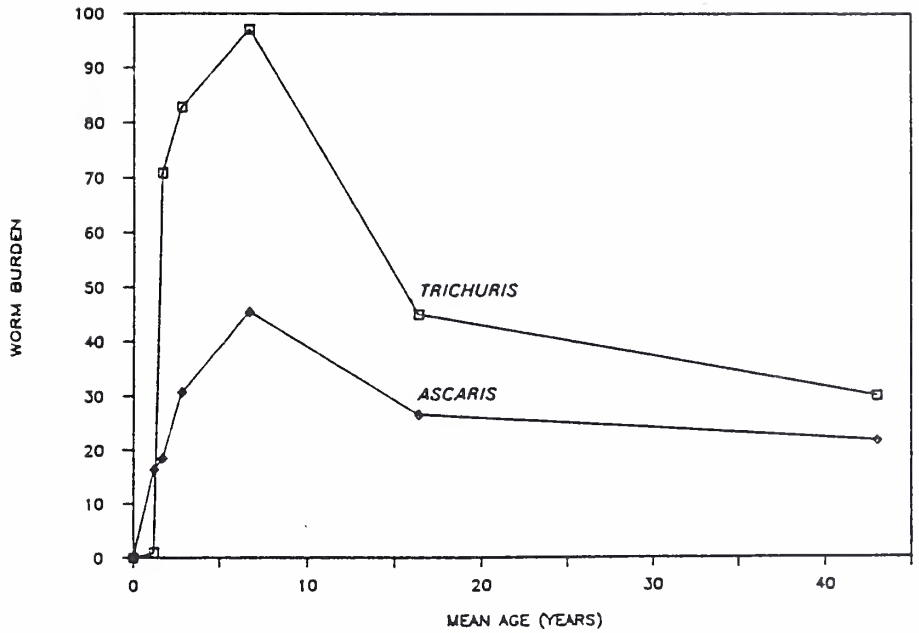


Figure 7. Age-intensity profiles for *Ascaris lumbricoides* and *Trichuris trichiura*. They are convex, attaining a maximum value in the 5-10 year age-class and declining in adulthood (*Ascaris* worm burdens are x7 to facilitate comparison) (Bundy 1990).

As a result, although individual hosts can be infected by all three parasites, their proportions will probably change as the hosts age.

As in hookworm, these other parasites also show an overdispersed pattern, often with 15% of the infected population harboring some 65% of the worm burden. The question arises whether, if a person has a tendency towards heavy hookworm infections, he or she would also have a tendency towards heavy infections by other geohelminths, or multi-species predisposition. Earlier discussion gave strong arguments for the existence of predisposition when looking strictly at hookworm infections, with equivocal evidence for its age-relatedness. Other studies solidly demonstrate predisposition to exist for other single helminth infections such as *Ascaris*, *Trichuris*, and *Enterobius vermicularis* (Bundy 1990, Anderson and Medley 1985). However, the evidence for multi-species disposition has been equally divided, and evidence in its support is weak at best (Bundy 1990).

In terms of egg production, the arguments for density-dependent fecundity may be extended into situations of multi-species infections, though mathematical models have not yet been demonstrated. The quantitative relationship between egg output per female worm and worm burden is species specific. *Trichuris* and hookworms show similar patterns and numbers, though egg production by *Ascaris* is generally an order of magnitude greater. Also, the mean worm burdens of hookworm and *Trichuris* tend to be similar, and an order of magnitude greater than *Ascaris* (Bundy 1990).

Southeast Asia:

There has been quite a bit of data produced from Asia and Southeast Asia where hookworm is endemic and considered to be a major public health

problem. However, much of this data is inaccessible and often times available only in the native language. Southeast Asia is populated by some 210 million people, with an estimated 30-70% being infected by hookworm (100 million). The predominant hookworm species in these areas is believed to be *N. americanus*, although species identification has often been omitted in studies of geohelminths. Marked variation in intensity and prevalence of hookworm has been found in many different localities, reflecting the strong influence of local environmental and behavioral patterns. It is also striking that the degree of co-infection with other geohelminths such as *Ascaris* and *Trichuris* varies widely between given areas. In contrast to the Philippines and Indonesia, where the prevalences of *Ascaris* and *Trichuris* generally run much higher than hookworm, hookworm seems to be the predominant geohelminth infection in Papua New Guinea (Carroll and Walker 1990).

Thailand is an ideal location for hookworm studies since much of the country is rural with generally high levels of hookworm infection. Two studies by Chongsuvavitwong *et al.* (1994, 1996), give some insight into the hookworm problem in southern Thailand. Prevalence of hookworm varies widely from province to province, ranging from 35-75% (Chongsuvavitwong *et al.* 1994). In terms of risk factors, the two studies gave conflicting evidence for the benefit of shoe-wearing, regular latrine use, and higher education. The latter study, an investigation of risk factors for hookworm transmission in 4 separate villages (Chongsuvavitwong *et al.* 1996), showed that only the wearing of shoes decreased the odds of acquiring hookworm. The other risk factors examined - regular latrine use, education and income levels - did not confer protection in the latter study but did so in the former (Chongsuvavitwong *et al.* 1994). Also conflicting with the earlier study (Chongsuvavitwong *et al.* 1994) was the finding that the risk for and intensity

of hookworm infection were not associated with either age or sex. As we have discussed earlier, hookworm transmission is very much an age-related phenomenon. Experiences with the hookworm problem in southern Thailand seem to be highly variable, and maybe location-dependent.

One factor to be eliminated from our list of risky behaviors is the use of human manure (nightsoil) for agricultural purposes. Unlike China, nightsoil is not routinely used in the fields surrounding Sangkhlaburi.

Immunology:

Threading throughout this discussion so far has been the questionable role of immunology on the patterns of hookworm transmission. The possibility of immunocompetence or immunocompromise in making a host more or less susceptible to infection has been strongly eluded to. The nature of host resistance has not been elucidated at the present. Earlier it was stated that the frequency distribution of hookworm in a population was highly overdispersed. It resembled a negative binomial distribution, with a small fraction of the study population harboring a large percentage of the total hookworm burden. At the tail end of the distribution curve, some studies even suggest a distinct cluster of people who had particularly high levels of infection. A thought about why certain people have higher levels of infection is that hosts who have some sort of immunocompromise (immature or poorly developed immune systems, immune systems impaired from other pathology such as malnutrition) may be more vulnerable to hookworm infection than their immunocompetent peers.

It is also unclear how immune status evolves as hosts age. In the previous section, it was reported that studies of age versus prevalence (or intensity) of infection showed a linear rises throughout childhood until a

peak was reached at approximately 20-30 years old, after which the prevalence (or intensity) reached a plateau or even fell slightly. An explanation for the concavity of some of these graphs is that repeated re-infection may induce some sort of immune response and acquired immunity by the host over time.

Many studies have been carried out to look for evidence of immunological activity caused by hookworm infection, and results have been equivocal. In one study, human volunteers were infected with low-dose *Necator*, and subsequent blood tests showed a transient eosinophilia and minor rise in IgE and IgG, but no other T cell nor B cell response (Maxwell *et al.*1987). It is possible that higher levels of infection were necessary to produce a detectable immunological change. On the contrary, a study by Pritchard *et al.* (1990a, 1990b)in Papua New Guinea measured the level of immune response (antibody level) to specific native hookworm proteins - the cuticular collagen of *N. americanus* and the excretory-secretory products - using ELISA on hosts 0-50 years old. Like other endemic areas, this study showed similar age versus intensity curves and a similar distribution (overdispersion) of hookworm among the hosts. Pritchard *et al.* found a significant positive correlation between levels of anti-collagen antibodies, egg count and age. When the effects of intensity were controlled, they also found significant positive correlation between age and antibody level. However, there was no significant correlation between intensity of infection and antibody level when the effects of host age were controlled, meaning there was no correlation between antibody level and degree of host resistance. These results indicate that immunity in adulthood is not regulated by exposure alone, but possibly by age factors and/or an acquired-type immunity.

The presence of antibodies in the host to hookworm cuticular collagen and ES products suggests that there must be other hookworm antigenic sites

or secretory proteins which will be able to induce an immune response. The Hotez lab has discovered and characterized a 42-kDa protein, *Ancylostoma*-Secreted Protein (ASP-1), which is released in-vitro by *A. caninum* larvae (L3) upon activation from the hypobiotic (arrested) state (Hawdon *et al.* 1996). Although activation was induced in the laboratory setting, the process is thought to occur also upon larval invasion of the host or after a period of dormancy. Continual, low-level release of ASP-1 is also thought to occur, maybe as a necessary protein for continued infectivity. ASP-1 has been cloned by the Hotez lab, and recombinant-ASP (rASP-1) produced. If ASP-1 is shown to induce an immune response, then rASP-1 may ultimately be a strong candidate for vaccine development.

Conclusion/suggestions for prevention:

Many lessons have been learned since the Rockefeller Foundation's first attempts to control the worldwide spread of hookworm in the early decades of the 1900's. Basic tenets about hookworm disease and transmission to be considered include the necessity to distinguish between those who are simply infected versus those who have clinical pathology secondary to infection; and to recognize that hookworm infection is a cumulative process resulting from repeated exposure, that sanitation alone is sufficient to control hookworm but is a very slow process, and that treatment of heavily infected people (whether it be a specific group or an entire community) can aid significantly in slowing the spread of hookworm.

Basically, the prevalence of soil-transmitted helminths reflects the socioeconomic status of the population (Gilles 1985, Pawlowski *et al.* 1991). The public health strategy often taken to control the spread of this parasite and its disease incorporates both short-term and long-term goals. Acutely, the

objective of public health intervention is to reduce the mortality and morbidity of hookworm disease. This is done on an individual basis by alleviating the effects of iron-deficiency and anemia via anthelminthic medications, iron supplements, proper nutrition, and blood products if necessary (Gilles 1985). Targeting treatment of groups of people with high levels of infection, or mass chemotherapy of entire communities, is the bridge between the short-term and long-term solution because it is prevention as well as cure. The long-term objective is simply to decrease and eventually eradicate the parasite completely within a community, and to treat the chronic disease of hookworm. This can be done by periodically targeting those with high levels of infection for anthelminthic medications or mass treatment (costly) if prevalence is extremely high, and providing long-term iron supplementation or teaching people how to improve their diets within a cultural context. More importantly, efforts should be concentrated on improving the general standard of living within a community. This includes more effective means of sanitary feces disposal, such as the construction and maintenance of sanitary latrines with piped water supply and adequate drainage of the waste. The importance of using clean water for cooking, rinsing vegetables, and washing dishes should be emphasized, as well as the need to wear shoes. A sufficient number of outhouses and alternatives to nightsoil as a fertilizer must be sought in agricultural production to eliminate the constant risk of re-infection when working in the fields. These measures demand a willingness to educate the community about public health and sanitation, and requires a degree of openness especially in dealing with potentially embarrassing topics such as defecation practices. Cultural mores and beliefs must be taken into consideration. Some countries, such as in Southeast Asia, are more open to discussion about these practices and there

already exist culturally prescribed defecation behaviors (Gilles 1985, Pawlowski *et al.* 1991). In essence, only with vigilance, improved sanitation, open communication, and efforts towards health education can a community truly rid itself of this disease.

Statement of Purpose

The purpose of this project was to investigate the epidemiology of human hookworm infections in the rural villages along the central Thai-Myanmar border, a task which had not been previous performed. More specifically, a basic assessment of prevalence, intensity, and distribution of hookworm infections was carried out. Identification of the predominant species (*Ancylostoma duodenale* or *Necator americanus*) in this area was important to further characterize the infections in this area. Cases of infantile hookworm infection were sought and investigated to look for evidence for vertical transmission. Common risk factors for hookworm transmission were explored, using questionnaires and calculations from the prevalence and intensity data. Lastly, an investigation into the nature of the immunological response to hookworm infection was initiated. The presence or absence of immunoglobulin formation to certain hookworm antigens, the level of response relative to the intensity of infection, and the level of response relative to age of the host were studied.

As well as being the basis of my thesis, this work will be the basis of masters thesis for Jeffrey Bethony, PhD candidate in epidemiology/biostatics at SUNY-Buffalo.

Methods and Materials

This project studies the basic epidemiology of the human hookworm, its distribution within the studied population, the nature of the host immunological response to hookworm infection, and how this response varies with age and with infection intensity. Ultimately, the results of this project may lay the groundwork for studies to evaluate potential vaccine candidates. Concurrently, the design of this project also allows us to indirectly investigate the possibility of vertical transmission by looking for infected mother-infant pairs, which would have a direct bearing on our knowledge of the natural history of hookworm and would be an important link in explaining the presence of ancylostomiasis in children.

This study was done in conjunction with Jeff Bethony, MA-PhD candidate in Epidemiology and Biostatistics at SUNY-Buffalo.

January 1996 - pilot study

Study site:

The study was conducted in an area located in central-western Thailand along the Thai-Myanmar border, 20 kilometers from a border-crossing known as "Three Pagoda Pass". The population studied was taken from the Sangkhlaburi, Nong Loo District, Kanchanaburi Province, Thailand. The Nong Loo District consists of thirty villages spread over a fifty mile range, with inhabitants of Mon, Thai, Karen, Burmese, and Laotian ethnic descent. Most residents are Burmese refugees who settled in the district following a decade of civil unrest then the eruption of the civil war in Burma in 1988. There continues to be significant emigration and immigration of families


BURMA
LAOS
VIETNAM
KAMPUCHEA
MALAYSIA
Chiang Rai
Fang
Muang Pakbang
Louangphrabang
Ban Ban
Thanh Hoa
Muang Xiagnabouri
Xiagnkhoang
Cua Rao
Mae Hong Son
Phayao
Chiang Mai
Nan
Muang Vangviang
Vinh
Ha Tinh
Mae Sariang
Muang Pakxan
Madauk
Phraa
Vientiane
Nong Khai
Ban Na Phao
Uttaradit
Loei
Udon Thani (Udorn)
Nakhon Phanom
Muang Khammouan
Sawankhalok
Sakon Nakhon
Thaton
Tak
Phitsanulok
Mukdahan
Savannakhat
Moulmein
Phetchabun
Khon Kaen
Roi Et
Khammarat
Nakhon Sawan
Bua Yai
Ubon Ratchathani
Pakxé
Ya
Sangkhlaburi
Lop Buri
Warin Chamrap
Sisaket
Nakhon Ratchasima (Korat)
Surin
Sara Buri
Muang Không
Nam Tok
Kanchanaburi
Phra Nakhon Si Ayutthaya
Kabin Buri
Andaman Sea
Ratchaburi
Bangkok
Aranyaprathat
Phnum Tbâng Méanchey
Stŏang Trâng
Samut Songkhram
Chon Buri
Sisŏphŏn
Siĕmréab
Phetchaburi
Bâtdâmbâng
Tonle Sap
Kâmpóng Thum
Sattahip
Rayong
Poŭthĭsăt
Krâchéh
Margui
Trat
Kâmpóng Chhnăng
Kampong Cham
Ko Chang
Ko Kut
Prachuap Khiri Khan
Krŏng Kaôh Kŏng
Phnom Penh
MERGUI ARCHIPELAGO
Kâmpóng Spoe
Svay Riĕng
Takêv
Kâmpot
Kâmpóng Saôm
Chumphon
Gulf of Thailand
Dao Phu Quoc
Can Tho
Ranong
Ko Phangan
Ko Samui
VIETNAM
Ca Mau
Takua Pa
Nakhon Si Thammarat
Ko Phuket
Phukat
Phatthalung
Strait of Malacca
Kantang
Trang
Songkhla
Hat Yai
Pattani
Ko Tarutao
Satun
Narathiwat
Kota Baharu
Pulau Langkawi
Alor Satar
Betung
Thailand
International boundary
National capital
Railroad
Road
0 50 100 150 Kilomatars
0 50 100 150 Miles
Boundary representation is not necessarily authoritative. Names in Vietnam are shown without diacritical marks.

into this area from just across the border. There are no official demographics or census type of data regarding the Nong Loo District.

There are two major seasons in Thailand, the wet monsoon season from June to October, and the dry season for the rest of the year. The vast majority of the yearly rainfall occurs during the monsoon season. The area around Sangkhlaburi and along the central Thai-Myanmar border is mountainous, and over 50% of the land is covered with tropical vegetation and forests.

The town of Sangkhlaburi sits on the opening of the Sangkaria River where it was dammed four years ago to create a man-made reservoir (known as Khao Laem Lake), and is therefore divided into two "halves" by the river/reservoir. Both a concrete bridge (for trucks) and a wooden suspension bridge (for motor scooters) between the two sides make transportation and communication continual and easy. The native Thais occupy the east half of Sangkhlaburi, which has a school, and small hospital, malaria center, a bank, two pharmacists, multiple hotels and guesthouses, and a main market. Most streets are asphalt-paved, and transportation consists of cars, trucks, and motorscooters. A Mon settlement occupies the west half of Sangkhlaburi, and most roads remain dirt-packed though main throughways are starting to become asphalt-paved. The roads and houses become more sophisticated farther from the water's edge, and were relatively very primitive along the water.

For this study, a section of the Mon settlement which lay along the bank of the reservoir was chosen. The inhabitants are predominantly of Mon origin, an ethnic group native to Myanmar. They live in bamboo/millet-stalk huts stilted approximate 2 feet above the ground, with chickens and stray dogs living underneath. Each hut contained a nuclear family usually

consisting of a mother, father, children, and sometimes parents of the mother or father. Huts were arranged in close rows along main dirt-packed *sois* (lanes).

Subject population and sample selection:

The goal of this study was aimed at determining the intensity, prevalence, and distribution of human hookworm in the studied population, as well as looking for evidence of vertical transmission of *A. duodenale* larvae from mother to breast-feeding infant. For this purpose, we used mother-infant pairs as our sampling unit. We defined "infant" as any child of age 24 months or younger. 108 mother-infant pairs were first sampled. From this population of pairs, the study was expanded to include other family members (father, siblings) of the original pairs. Individuals ranging in ages 0-65 years were included. As many other family members were included given our time constraints and supply limits.

Stool samples were collected from each participant.

Stool analysis:

Each participant in the study was given an 8-ounce styrofoam ice cream cup and tongue depressor, and was told to submit an "egg-size"' sample of stool which had not touched the ground. The stool samples were collected within 24 hours of defecation (daily visits to the households for the next 3-4 days) and kept on ice at 10-15°C until they could be analyzed in our laboratory - within 6 hours of collection of the samples or maximum of 24 hours if there was an electric black-out.

The stool samples were analyzed using a version of the McMaster's saline-float technique for quantitative egg counts of hookworm, *Ascaris*, and

Trichuris. The stool was stirred in the collection cup with a wooden tongue depressor until it was homogenous in consistency. Two grams of stool were weighed on weigh paper using an electronic scale, then mixed thoroughly with 60 ml 35% NaCl (supersaturated saline). The stool-saline solution was the strained through a sieve twice to remove all particulate matter. The remaining solution was stirred vigorously while being transferred to 15 ml conical tubes, which were used for transport to the microscope area.

At the microscope area, the solution was mixed thoroughly by pipet to re-homogenize the solution, since the hookworm eggs would have been suspended at the top of the solution. The solution was then pipetted into an original McMaster's egg-counting slide - a slide with two cells, each cell marked with a counting grid. Eggs for hookworm (species indistinguishable), *Ascaris*, and *Trichuris* were counted using a light microscope under 10x power. The egg count from the 2 cells were added, then multiplied by 100 to obtain the measurement "eggs per gram feces".

Summer 1996

Overview:

The goals of this study were aimed at advancing the information obtained from the pilot study about the epidemiology of the human hookworm in the studied population and about the possibility of vertical transmission of *A. duodenale* larvae from mother to breast-feeding infant. Furthermore, the study was designed to explore in-depth some of the risk factors involved in the transmission of hookworm and other gastrointestinal helminths, and to begin to gather serological evidence for hookworm infection.

We visited several sites (small and large villages) where hookworm infection is endemic, and sampled in family groupings. We collected both a stool and a blood sample from children (blood from children over the age of 2 years) and adults. Blood was collected by venipuncture or fingerstick in adults, and by fingerstick or heelstick in children. Quantitative egg counts (McMaster technique) were performed on all stool samples obtained to determine intensity of infection. The species of hookworm was to be determined by microscopic examination of the third-stage larvae reared from the eggs, though PCR-RFLP proved to be more successful. Serum was isolated from all blood samples using centrifugation, then frozen and transported to the United States (SUNY-Buffalo) for immunological analysis (ELISA).

All infected individuals under 12 years of age were treated with pyrantel pamoate, and those 12 years old or older with mebendazole.

Study sites:

The populations studied were taken from three distinct rural areas neighboring Sangkhlaburi, which were labeled appropriately as Villages 1, 2, and 3.

Villages 1, 2, and 3 have approximately the same amount of rainfall as the pilot study population in the town of Sangkhlaburi. These refugee settlements were located at a distance from the main town of Sangkhlaburi, so therefore smaller and more rural. Conditions in the two Mon settlements (Village 1 and 3) were primitive, with more distantly spaced huts with no or makeshift outhouses, narrower *sois* (lanes), and agricultural animals (pigs, cattle, chickens) roaming freely about the village. The Karen settlement

(Village 2), although the farthest removed from the town of Sangkhlaburi, was the most developed, with many wide, concrete paved roads.

Village 1 - This village consists of approximately 55 households, and is the remnant of a previously larger village which was flooded four years ago in an attempt to create a man-made water reservoir. This village sits on the bank of that reservoir. Most residents are of Mon ethnic descent, and all spoke Mon with some to little knowledge of Thai.

Village 2 - This village consists of 200 households. This village consists of people of mostly Karen ethnic descent, and all spoke both Karen and Thai.

Village 3 - This village consists of approximately 250 households, mostly of Mon ethnic descent. A major roadway ran through the center of the village.

Subject population and sample selection:

From pilot studies performed in January 1996, we were able to estimate the prevalence of hookworm for different age groups in the region. From this data, we determined that approximately 150 households (average of 5 members/household, or approximately 750 individuals total, ranging from 0-100 years of age) was an ideal sample size for this study. Considering our length of stay and physical constraints, the cohort was still large enough to statistically minimize variance, and to look for genetic/sociological/immunological patterns within the family groupings. We estimated that, of 150 households, approximately 120 households would have children under the age of 24 months.

Village 1 - As stated earlier, this village consists of approximately 55 households. We attempted to include in the study every member of every

household in this village, and were able to obtain stool samples from 55 households and 196 residents.

Village 2 - We sampled all households along 2 major concrete-paved *sois*, which totaled 29 households and 159 residents.

Village 3 - We sampled a total of 99 households - 65 households which constituted all households on one side of the major roadway running through the village, and 34 households from 3 major dirt *sois* on the other side of the roadway. The total number of residents sampled for stool was 305.

Screening questions for pregnancy were asked of all women of child-bearing age, and those women with high-probability of pregnancy were excluded from the study. Before being asked to participate, all candidates were screened and briefly examined for evidence of acute medical problems.

At each village, the head of the village was first asked whether he would be interested in having his village participate. The project goals, procedures, and risk factors were explained in detail to him. After his consent, we proceeded to go door-to-door, explaining the details of the study and asking each household head whether he/she was interested in having his/her family participate. Each household was admitted into the study after verbal consent of the head(s), usually the mother and/or father. Informed consent was obtained orally (accordingly with the approved Human Investigations Committee protocol #8881), since 90-95% of the household heads were illiterate.

For each household, a questionnaire was administered. Stool samples were collected from every compliant member of the household. Blood samples were collected from those over the age of 24 months with his/her consent. Individuals and households could refuse to participate at any time during the project.

Questionnaire:

To obtain information about each household concerning the names and ages of each member of the household, level of education, occupation, and general socioeconomic status, a questionnaire (next page) was administered to the head(s) of the household, usually to the mother and/or father. The questionnaire explored risk factors for gastrointestinal parasites - size and layout of the household, types of material from which the floors and household was constructed, the presence or absence of bathroom facilities, the construction of the outhouse, electricity and presence of electrical appliances, water sources, previous anthelminth treatment, use of shoes, and basic questions concerning pregnancy and birth rates.

Questionnaires were administered in Mon in Villages 1 and 3, and in Thai in Village 2. They were administered by a native Mon young woman who lived in the central Mon settlement in Sangkhlaburi, and who was supervised by the research team. Questionnaires were completed with most family members being present, and were addressed to the head(s) of the household. Answers were written in Thai then translated.

Stool analysis:

Stool analysis and hookworm egg counts were performed by the same technique which was used during the pilot study (see above).

If >1000 eggs per gram feces were found in the stool sample, the stool was then "cultured" to grow out the actual hookworm larvae. Since so many individuals were found to have hookworm eggs in their stool, this cut-off value was decided arbitrarily considering our supply and time limitations. The stool was cultured using two techniques - the Harada-Mori technique and the Baermann technique - in order to rear third-stage larvae for microscopic

Questionnaire

1. Reference name
2. Reference age
3. Ethnic group: Mon, Burmese, Karen, or Thai
4. How many pregnancies
5. How many live births
6. How many light fixtures in the house
7. Do you own a t.v.
8. Do you own a refrigerator
9. How many rooms in the house
10. How many rooms have dirt floors
11. How many rooms have bamboo floors
12. How many rooms have wood floors
13. How many rooms have cement floors
14. Do you have a bathroom
15. If you do not have a bathroom then where do you go: neighbor, communal, forest, bank of a river, or other
16. Where to children under the age of 2 stool: earthen floor, wooden floor, crib, diapers, or bathroom
17. If you use diapers, how often are they changed
18. What is the floor of the bathroom made of: earth, split bamboo, cement, or tile
19. (in Thai)
20. (in Thai)
21. Father's occupation: swidden rice farmer, gardener, wet rice farmer, fisherman, wood cutter, day laborer, vegetable seller, merchant, or other
22. Mother's occupation: housewife, gardener, wet rice farmer, fisherman, wood cutter, day laborer, vegetable seller, merchant, or other
23. (in Thai)
24. (in Thai)
25. (in Thai)
26. Where do you wash your clothes: bathroom, well, riverbank, or canal
27. (in Thai)
28. How often do people in this house wear shoes: never, sometimes, always
29. (in Thai)

identification of species (*A. duodenale* vs. *N. americanus*) and to produce a hookworm antigen against which the enzyme-linked immunosorbent assays (ELISA's) would be performed. The Baermann technique is a more effective technique for producing larger quantities of hookworm larvae. However, it requires more equipment, supplies, and buffer than the Harada-Mori technique, and we were limited in all three. Therefore, both techniques for harvesting larvae were used.

Each participant in the study was given an 8-ounce styrofoam ice cream cup and tongue depressor, and was told to submit an "egg-size"' sample of stool which had not touched the ground. The stool samples were collected within 24 hours of defecation (daily visits to the households for the next 3-4 days) and kept on ice at 15°C until they could be analyzed in our laboratory - within 6 hours of collection of the samples or maximum of 24 hours if there was an electric black-out.

The stool samples were analyzed using a version of the McMaster's saline-float technique for quantitative egg counts of hookworm, *Ascaris*, and *Trichuris*. The stool was stirred in the collection cup with a wooden tongue depressor until it was homogenous in consistency. 2 grams of stool were weighed on weigh paper using an electronic scale, then mixed thoroughly with 60 ml 35% NaCl (supersaturated saline). The stool-saline solution was the strained through a sieve twice to remove all particulate matter. The remaining solution was stirred vigorously while being transferred to 15 ml conical tubes, which were used for transport to the microscope area.

At the microscope area, the solution was mixed thoroughly by pipet to re-homogenize the solution, since the hookworm eggs would have been suspended at the top of the solution. The solution was then pipetted into an original McMaster's egg-counting slide - a slide with two cells, each cell

marked with a counting grid. Eggs for hookworm (species indistinguishable), *Ascaris*, and *Trichuris* were counted using a light microscope under 10x power. The egg count from the 2 cells were added, then multiplied by 100 to obtain the measurement "eggs per gram feces".

If hookworm was present, the stool was then cultured using both the Harada-Mori technique and the Baermann technique in order to rear third-stage larvae for microscopic identification of species (*A. duodenale* vs. *N. americanus*) and to produce the hookworm antigen against which the enzyme-linked immunosorbent assays (ELISA's) would be performed. The Baermann technique is a more effective technique for producing larger quantities of hookworm larvae. However, it requires more equipment, supplies, and buffer than the Harada-Mori technique, and we were limited in all three. Therefore, both techniques for harvesting larvae were utilized.

The harvested larvae were then pooled and cleared of impurities using a PBS buffer filter-column, and concentrated in PBS buffer into pellets stored at -20°C until transport back into the United States.

Harada-Mori culturing technique

Whatman filter paper was cut into strips 4 cm x 18 cm, then bent in half lengthwise. Approximately 2 grams of stirred stool was spread onto the inner side of the filter paper using a wooden tongue depressor, sparing 3 cm and 1 cm on respective ends. 20 in x 1.5 in diameter Kimex glass tubes were filled with 1 inch of distilled H_2O, and the smeared filter paper strip was inserted into the tube with the unsmeared 3 cm at the bottom. The water level in the tube was not allowed to touch the stool on the filter paper. The openings of the tubes were all covered twice with parafilm, and airholes (vital) were poked into the parafilm. The tubes were labeled, then incubated

upright in a warm (20-30°C) dark cupboard for 6-8 days. The water level at the bottom of the tube was checked daily for evaporation and maintained at a level to cover the bottom of the filter paper.

Starting day 5, the glass tubes were manipulated under a stereoscope and examined for the presence of hookworm larvae which had hatched in the stool and migrated down the filter paper into the water. The larvae were then harvested and pooled by pouring the water into 15-ml conical tubes. The larvae was loosely pelleted with brief centrifugation, and the pellet washed 2-3x in PBS buffer in an effort to remove as much particulate matter as possible, then sent through the PBS buffer filter-column (see below) to concentrate them further and prepare them for storage and freezing.

Baermann culturing technique

Stool positive for hookworm ova was mixed with approximately 2 to 3 times its volume with bone charcoal (sphagnum moss could also be used) and a minimal amount of distilled water to make a very thick, black, mudpie-like sludge. Plastic disposable 3-inch diameter petri plates were lined with a piece of round Whatman filter paper and the filter paper moistened with a squirt of distilled water. The stool/charcoal mixture was then scooped evenly into the petri plates and the lids replaced. The plates were then labeled, stacked, and incubated in a warm (20-30°C) dark cupboard for 6-8 days.

Starting day 5, the petri plates were opened and examined under the stereoscope for the presence of hookworm larvae moving along the surface of the mixture. The lid was also examined for larvae, since they migrated into the condensation water droplets formed on the lid. The larvae from the lids were washed directly into the PBS buffer filter-column for cleaning and concentration. The worms moving along the surface of the charcoal were

harvested using a combination filter/funnel apparatus. A 5-inch diameter circle of steel sieve was attached to a lucite ring of the same diameter to act as a filter. This sieve was lined with two Kimwipes, then the charcoal mixture scooped into this. The sieve was placed into the mouth of a large funnel. A one-foot long rubber tubing was attached to the neck of the funnel and clamped near the free end. The funnel and tubing were filled with distilled water until the level just covered the charcoal sludge. The principle behind this apparatus lies in the fact that larvae migrate downward through the Kimwipes and settle at the bottom of the tubing, filtering out the charcoal sludge. After 24 hours at room temperature, the larvae were skimmed from the bottom of the tubing, then poured into the PBS buffer filter-column for further concentration and freeze storage.

PBS buffer filter-column:

This apparatus works on similar principles to the filter/funnel described above. A piece of flexible metal wide-holed mesh was sandwiched between two stacked plastic beakers whose bottoms had been cut out. The beakers were then placed into a cylindrical funnel with tubing attached to the neck and clamped at the end. The funnel was filled to above the level of the mesh with PBS buffer. With a piece of Kimwipe lining the inner beaker, the harvested larvae were poured into the beaker and allowed to sit for a few hours (4-5) at room temperature until they could be detected at the bottom of the tubing.

Larvae were drained from the tubing, then gently centrifuged to create a larval pellet. Larval pellets in PBS were stored in 1.5-ml Eppendorfs at -20°C until transport back to the United States.

Blood samples:

Blood samples were obtained from every consenting member of the household age 2-65 years who had registered for the study, using fingersticks or heelsticks for children under the age of 10, and venipuncture or fingersticks for those older than 10 years. We collected approximately 300-600 µl using the Microtainer collecting system (Becton-Dickinson, Bangkok, Thailand) from those with finger- or heelsticks, which consisted of an open tube with a scooping device at the opening. Venipuncture samples were drawn into 10-ml red-top Vacutainer (Becton-Dickinson, Bangkok, Thailand) tubes with no additives. Blood samples were carried on ice in the field until the end of the day, when they were refrigerated overnight at 40°C to allow for further clotting. Samples were then centrifuged, and the serum was poured into sterile 1.8-ml Cryotubes, taking care to avoid the Buffy coat and the red blood cells not compressed into the clot. Transferring the serum was performed using sterile technique and a flame. Serum was frozen at -20°C, then transferred to -80°C for the duration of the international work. The serum was transported in a specially insulated and sealed field carrier to the SUNY-Buffalo lab for immunological analysis via enzyme-linked immunosorbent assays (ELISA's). Serum was tested for the presence and level of IgG (subclasses 1, 2, and 3), IgM, IgA, and IgE (isolated via Sephadex columns) against antigenic proteins derived from the frozen hookworm larvae and from Dr. Hotez's recombinant ASP protein, a protein targeted for its possible immunogenic properties. We did not obtain blood samples from children under the age of 2 years, because of their nonstandard immunology (especially the infants) and an unwillingness to subject them to discomfort.

Treatment:

All children under the age of 12 years harboring intestinal parasites (hookworm, *Ascaris*, *Trichuris*) were treated with pyrantel pamoate (11 mg/kg x 1 dose (max. 1 gr/day)). All subjects age 12 years or older with intestinal parasites were treated with mebendazole (100 mg bid x 3 days).

Species determination of pooled hookworm larvae via PCR-RFLP of genomic DNA:

Hookworm genomic DNA was isolated from the transported pooled hookworm larvae and DNA concentration determined by spectrophotometer. This work took place in the SUNY-Buffalo lab (Jeff Bethony) using standard DNA extraction protocols.

PCR of this DNA was performed in the Hotez lab of Yale University using a protocol described by Hawdon *et al.*, 1995 and Hawdon 1996. A 473-bp fragment (long fragment or LF) of the 3′ untranslated region (UTR) of the cDNA encoding the catalytic subunit of an *A. caninum* cAMP-dependent protein kinase (PKA; GenBank accession number U15983) was amplified by PCR, using the primers PKA 5′-4 (5′- CTCGCCAGCATCTCTGCG - 3′) and PKA 3′-1 (5′- GCCTACAAACTTGTCAGCAGG). PCR reactions contained 100 ng of each primer, 100 ng of template DNA, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, and 1 unit of Taq DNA polymerase (Promega, Madison, Wisconsin) in a 20-µl reaction volume. 15 µl mineral oil was overlaid on the samples before PCR. For our experiments, template DNA included the DNA isolated from the pooled hookworm larvae as well as DNA from laboratory stocks of *A. duodenale*, *N. americanus*, *A. caninum* as controls.

The reactions were subjected to 30 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C. Reaction products (15%) were resolved on a 2% agarose/TAE gels containing 0.5 mg/ml ethidium bromide. *N. americanus* and *A. duodenale* long fragment DNA are indistinguishable on agarose gels. Amplification of hookworm genomic DNA using primers derived from the 3'-UTR of the *A. caninum* PKA cDNA yielded a single band of approximately 470 bp - the long fragment.

Subsequently, 5µl of each PCR reaction was subject to restriction enzyme digestion and RFLP determination. TaqI was chosen for its known recognition sites within the *A. caninum* PKA 3'-UTR long fragment (Hawdon *et al.*, 1995) and within the *A. duodenale* and *N. americanus* long fragment (Hawdon, 1996). Both *Ancylostoma* species have 3 TaqI sites, while *Necator* has only 1 TaqI site. Restriction enzyme digests were resolved on 7.5% polyacrylamide gels in TBE buffer, stained with ethidium bromide, and visualized with UV light.

Mixing experiments were performed to determine, in the presence of dual-species hookworm infection in the villages, the extent which the PCR-RFLP test would be able to detect the presence of both species, even if the ratio of the *Americanus*:*Necator* was extremely unbalanced. For mixed DNA experiments, pooled larval DNA:*A. duodenale* (chosen after initial experiments) ratios of 100:10, 100:1, and 100:0.1 were approximated by mixing appropriate amounts of the respective genomic DNA's. The total hookworm DNA in each PCR reaction was approximately 100 ng.

Epidemiology and biostatistics:

All biostatistics were done on the SPSS biostatistical analysis program. Comparisons of levels of hookworm infection (prevalence) and intensity of

infections between various groups was performed using t-tests, chi-square, odds ratios, and ANOVA. Statistically significant relationships between positive infections and risk factors such as age, sex, ethnic group, village, bathroom type, shoe-wearing, and occupation were sought. Reported *P* values are two-tailed unless otherwise indicated.

Results

The January 1996 studies of intensity, prevalence, and distribution of hookworm and other intestinal nematodes were pilot studies, whose results were used to guide the more extensive experiments of the summer 1996. The experiments performed during the summer was a further investigation into intensity, prevalence, distribution, and risk factors of hookworm transmission. In addition, the nature of the immunological response was investigated, in the effort to look for evidence of acquired immunity to hookworm infection. Specifically, the presence or absence of immunoglobulin formation to certain hookworm antigens, and its correlation to the intensity of infection were investigated.

The results of the immunological studies are not presented here, since they are still in their preliminary stages.

Hookworm species identification:

For the January (pilot) study, the larval samples which were transported back to the United States were not sufficient to identify the species of hookworm infecting our study subjects. However, we were able to harvest enough larvae during the summer experiments to at least identify the hookworm species predominant during this time. It was necessary to determine the species of hookworm early in our data analysis. Since infection by the two hookworm species can have very different characteristics, early determination of the species directed further thinking and calculation. We did not intend to identify the species of hookworm infecting individuals,

so the hookworm larvae were pooled in the field during cleaning and concentration with the PBS filter-column. Our goal was to determine which species (*A. duodenale* and/or *N. americanus*) were present, and their general proportions. Of note, only those individuals with hookworm egg counts >1000 eggs per gram stool were included in the production of the hookworm larvae pool. In the end, this resulted in 35% of all the subjects with hookworm infections contributing to the pool.

From PCR-RFLP, the pooled larvae gave a restriction pattern exclusive for *N. americanus*, with no visible evidence *of A. duodenale* being present (Figure 9). This finding is further strengthened by the fact that *A. duodenale* has a tendency to amplify better than *Necator* during PCR, and is therefore *more* likely than *N. americanus* to appear.

To explore whether even very small amounts of *A. duodenale* DNA could be detected if it was present in the pooled larvae, we performed mixing experiments where various amounts of the two species were mixed, then tested with PCR-RFLP. The tested ratios of pooled larvae:*A. duodenale* (in ng) were 100:10, 100:1, and 100:0.1, as well as unmixed (pure) samples. Figures 10 and 11 show the results of both the agarose gel after PCR amplification, and the PAGE after enzyme restriction. In these mixed samples, there is actually only little A. duodenale present even after PCR and enzyme restriction. It is possible that some sort of inhibitory effect or shielding interaction takes place when the two types of DNA are mixed, since multiple attempts at this experiment showed similar problems in producing evidence of the existence of *A. duodenale* DNA.

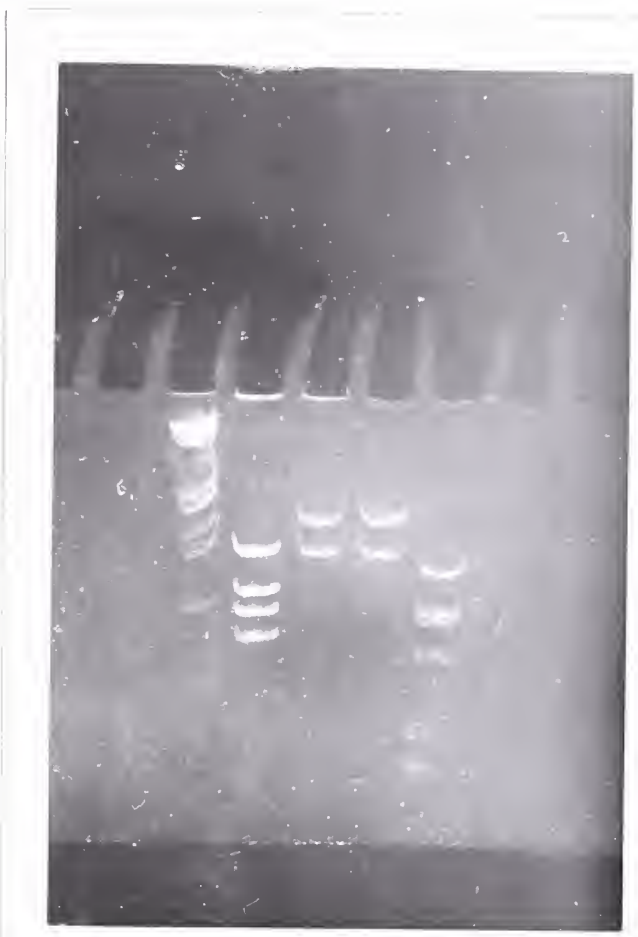
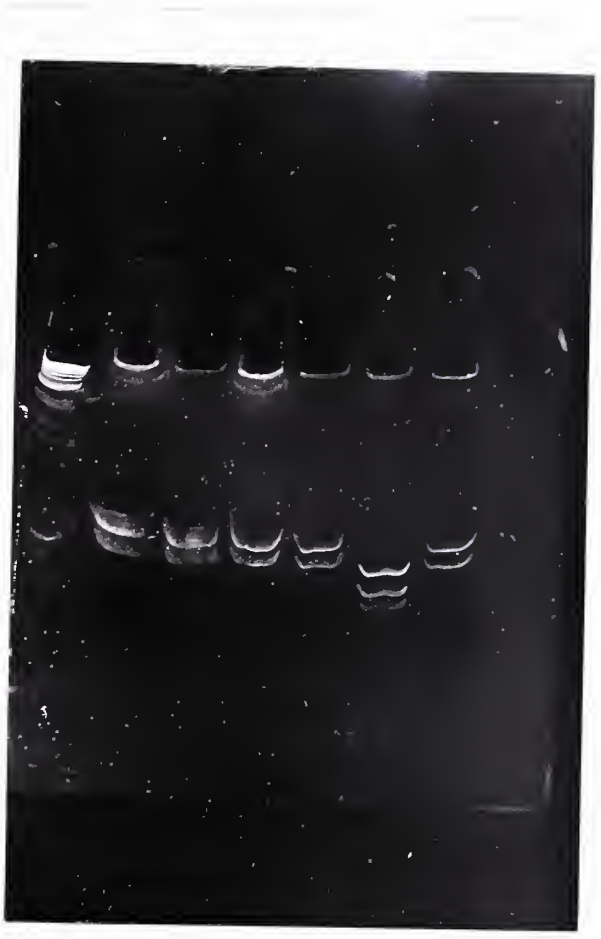


Figure 9. Species determination by PCR-RFLPs. The LF region was amplified by PCR from 50 ng genomic hookworm DNA, digested with Taq I restriction enzyme, separated on a 7.5% polyacrylamide gel, and stained with ethidium bromide. Lane 1 - 1 kilobase marker, lane 2 - *A. duodenale*, lane 3 - experimental pooled larvae, lane 4 - *N. americanus*, lane 5 - *A. ceylanicum.*



Figures 10 and 11. Mixing studies of hookworm DNA. Experimental pooled larvae:*A. duodenale* genomic DNA were mixed in ratios of 100:10, 100:1, and 100:0.1, and used as templates for PCR to amplify the LF region. The PCR product (LF region) was run on a 2% agarose gel to ensure amplification, then digested with TaqI restriction enzyme, separated on a 7.5% polyacrylamide gel, and stained with ethidium bromide. Each PCR reaction contained 100 ng genomic DNA from the experimental larvae plus the appropriate amounts of *A. duodenale* genomic DNA. Figure 10 is the 2% agarose gel (stained with ethidium bromide) after PCR amplification of the LF fragment. Figure 11 is the polyacrylamide gel after enzyme restriction with Taq I. Lane 1 - 1 kilobase marker, lane 2 - 100:10 experimental larvae:*A. duodenale*, lane 3 - 100:1 experimental larvae:*A. duodenale*, lane 3 - 100:0.1 experimental larvae:*A. duodenale*, lane 4 - *A. duodenale*, lane 5 - *N. americanus*.

Study group:

Pilot study - in the confined boundaries of the Nong Loo District of Sangkhlaburi, Thailand, we attempted to get every infant under the age of 24 months and his/her mother enrolled in the study, with 100% participation from every mother asked. 108 mother-infant pairs were first recruited and their stool samples analyzed. We then returned to the families of these pairs to recruit the other family members. In total, we analyzed the stool samples of 373 people - 199 female (53.4%) and 174 male (46.6%).

Summer study - from 3 villages surrounding Sangkhlaburi, Thailand, we were able, initially, to obtain almost 100% participation from all of those we asked: only one household in Village 3 refused to register for the study and to answer the questionnaire. The residents of the village were enthusiastic to participate, since treatment would follow if the individual was infected. A total of 869 men, women, and children were registered for the study, taken from a total of 182 households (Figures 12, and Table 1). Ages ranged from 1 month to 85 years in age.

Table 1. Summer study. Age profile of registered subjects (includes those who were away on jobs). N=804

	Male			Female			Total		
Age (yrs)	N	%	Cum%	N	%	Cum%	N	%	Cum%
0-2	32	8.4	8.4	44	10.4	10.4	76	9.5	9.5
3-5	33	8.6	17.0	52	12.3	22.7	85	10.6	20.1
6-10	63	16.5	33.5	51	12.1	34.8	114	14.2	34.3
11-15	34	8.9	42.4	43	10.2	45.0	77	9.6	43.9
16-20	20	5.2	47.6	32	7.6	52.6	52	6.5	50.4
21-25	28	7.3	54.9	34	8.1	60.7	62	7.7	58.1
26-30	33	8.6	63.5	40	9.5	70.2	73	9.1	67.2
31-35	37	9.7	73.2	22	5.2	75.4	59	7.3	74.5
36-40	26	6.8	80.0	35	8.3	83.7	61	7.6	82.1
41-45	15	3.9	83.9	20	4.7	88.4	35	4.4	86.5
46-50	15	3.9	87.8	11	2.6	91.0	26	3.2	89.7
51-55	12	3.1	90.9	8	1.9	92.9	20	2.5	92.2
56-60	11	2.9	93.8	12	2.8	95.7	23	2.9	95.1
61-65	13	3.4	97.2	9	2.1	97.8	22	2.7	97.8
66+	10	2.6	99.8	9	2.1	99.9	19	2.4	101.2
Total	382	100.0		422	100.0		804	100.0	

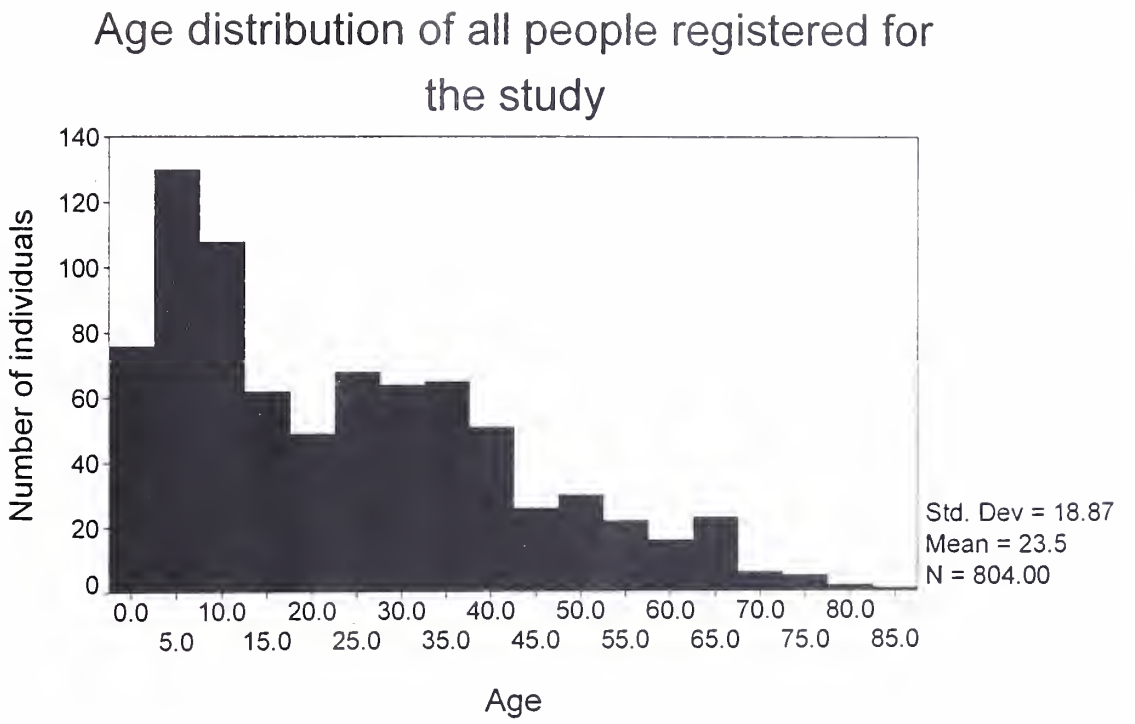


Figure 12. Summer study. Age profile of all those who registered for the study and completed the questionnaire (with a recorded age). N=804

Since questionnaires were performed by household units, all 869 people were, in effect, included in the completion of this questionnaire. Those who were seriously ill or known to be pregnant were then screened out. From the remaining group, 660 people (age 1 month to 85 years) gave stool samples for analysis. The remaining 209 were noncompliant or left the village for work before the study was completed (see section on Those who did not participate in the study). Of the 660 subjects giving stool samples, 367 were female (55.6%) and 293 were male (44.4%). This sex ratio was consistent for the individual villages. Of the 660 subjects submitting stool, 196 (29.7%) came from Village 1, 159 (24.1%) from Village 2, and 305 (46.2%) from Village 3.

Of these 660 subjects, we were able to obtain blood samples from 555 of those who were over the age of 24 months. The remainder were either too young, had taken anthelminthic medications within the past year (often administered at school, especially in the Karen village (Village 2)), or refused to give a blood sample.

Age distribution of the study groups:

Pilot study - Since the structure of the study was originally aimed at obtaining mother-infant pairs, the age profile of the studied population was heavily weighted towards children under the age of 2 years and females (the mothers) of child-bearing age (oversampling for infants and young mothers).

Table 2. Pilot study. Age profile of participants. N=373

	Male			Female			Total		
Age (yrs)	N	%	Cum%	N	%	Cum%	N	%	Cum%
0-2	66	37.9	37.9	42	22.0	22.0	108	29.0	29.0
3-5	18	10.3	48.3	15	7.7	29.7	33	8.8	37.8
6-10	26	14.9	63.2	29	14.9	44.6	55	14.7	52.5
11-15	13	7.5	70.7	16	8.2	52.8	29	7.8	60.3
16-20	5	2.9	73.6	9	4.6	57.4	14	3.8	64.1
21-25	6	3.4	77.0	27	13.8	71.2	33	8.8	72.9
26-30	11	6.3	83.3	23	11.8	83.0	34	9.1	82.0
31-35	14	8.0	91.3	19	9.5	92.5	33	8.8	90.8
36-40	8	4.6	95.9	10	5.0	97.5	18	4.8	95.6
41-45	3	1.7	97.6	5	2.5	100.0	8	2.1	97.7
46+	4	2.3	99.9	-	0.0	100.0	4	1.1	98.8
Total	174	100.0		199	100.0		373	100.0	

Summer study - the age profile of all those registered in the study (N=889) and those who actually submitted a stool sample (N=660) (Figures 12 and 13) for this study were very similar. Table 1 above shows the distribution of all those registered for the study, while the table below (Table 3) shows the distribution of the 660 who submitted stool. A large proportion of both populations fell between the ages of 0 and 15 years. Approximately 45% of the people who submitted stool samples fell under the age of 15, and 80% were age 40 or younger. Noticeably, there is a relative paucity in number of subjects in the 16-30 year old age range, most likely because they left the village during the day to work.

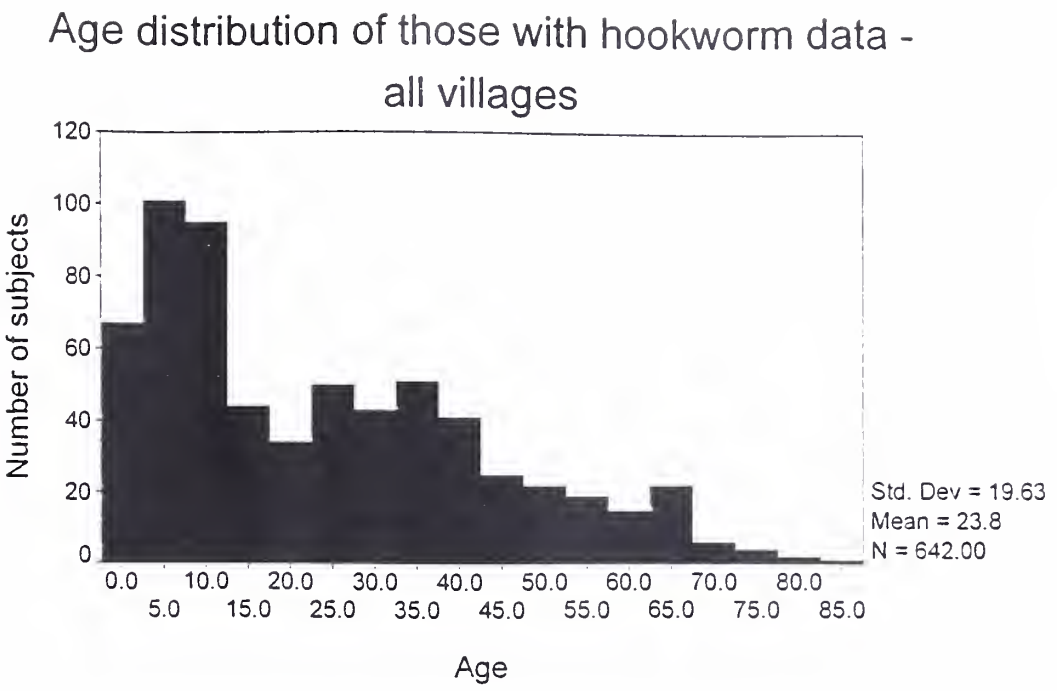


Figure 13. Summer study. Age profile of all subjects submitting a stool sample (for whom we have hookworm data) and an age recorded. N=642

Table 3. Summer study. Age profile of those submitting stool samples (for whom we have hookworm data). N=660

	Male			Female			Total		
Age (yrs)	N	%	Cum%	N	%	Cum%	N	%	Cum%
0-2	30	10.6	10.6	37	10.3	10.3	67	10.4	10.4
3-5	22	7.8	18.4	45	12.5	22.8	67	10.4	20.8
6-10	48	17.0	35.4	44	12.2	35.0	92	14.3	35.1
11-15	27	9.6	45.0	39	10.8	45.8	66	10.3	45.4
16-20	13	4.6	49.6	22	6.1	51.9	35	5.5	50.9
21-25	17	6.0	55.6	26	7.2	59.1	43	6.7	57.6
26-30	17	6.0	61.6	34	9.4	68.5	51	7.9	65.5
31-35	23	8.2	69.8	20	5.6	74.1	43	6.7	72.2
36-40	19	6.7	76.5	31	8.6	82.7	50	7.8	80.0
41-45	15	5.3	81.8	18	5.0	87.7	33	5.1	85.1
46-50	12	4.3	86.1	8	2.2	89.9	20	3.1	88.2
51-55	8	2.8	88.9	6	1.7	91.6	14	2.2	90.4
56-60	11	3.9	92.8	12	3.3	94.9	23	3.6	94.0
61-65	11	3.9	96.7	9	2.5	97.4	20	3.1	97.1
66+	9	3.2	99.9	9	2.5	99.9	18	2.8	99.9
no age	11			7			18		
Total	293	100.0		367	100.0		660	100.0	

Hookworm distribution:

In both the pilot and summer studies, the intensity of hookworm infections followed that of a negative binomial distribution (Figure 14 for the summer study), in which most infections were light infections (<1000 eggs per gram feces). The overall prevalence of mixed hookworm infections in the pilot study was 37.4% across all age groups, and overall prevalence in the summer study was 57.3% across all age groups. This disparity can probably be attributed to the fact that these studies were performed in different locales (though the socioeconomic conditions found in the two studies were similar), but seasonal variation in hookworm infectivity should also be considered.

The distribution of hookworm infection is highly aggregated and overdispersed, and follows a negative binomial curve. In the summer study, 65.8% of the people had light infections (<1000 eggs per gram stool). In terms of heavy infections, 2.4% of the subjects produced 19.2% of the hookworm eggs, and 14.8% of the subjects held 55.5% of the hookworm burden (as

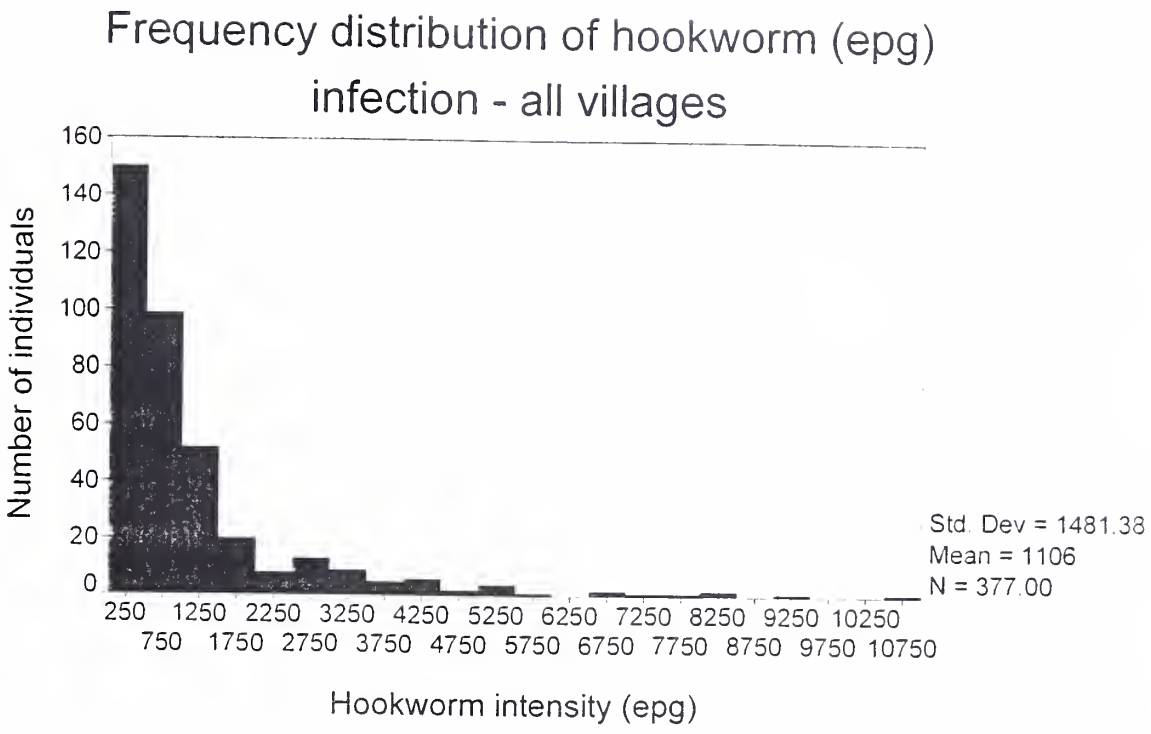


Figure 14. Summer study. *N. americanus* intensity (eggs per gram stool) histogram of all subjects with positive hookworm egg counts. The histogram shows a negative binomial distribution. 65.8% of hookworm-infected subjects had light infections (<1000 eggs per gram stool), while 14.8% produced 55.5% of the hookworm eggs. Includes all subjects with positive hookworm egg counts. One case, a 3 year old female with 19,800 epg, was eliminated. N=378

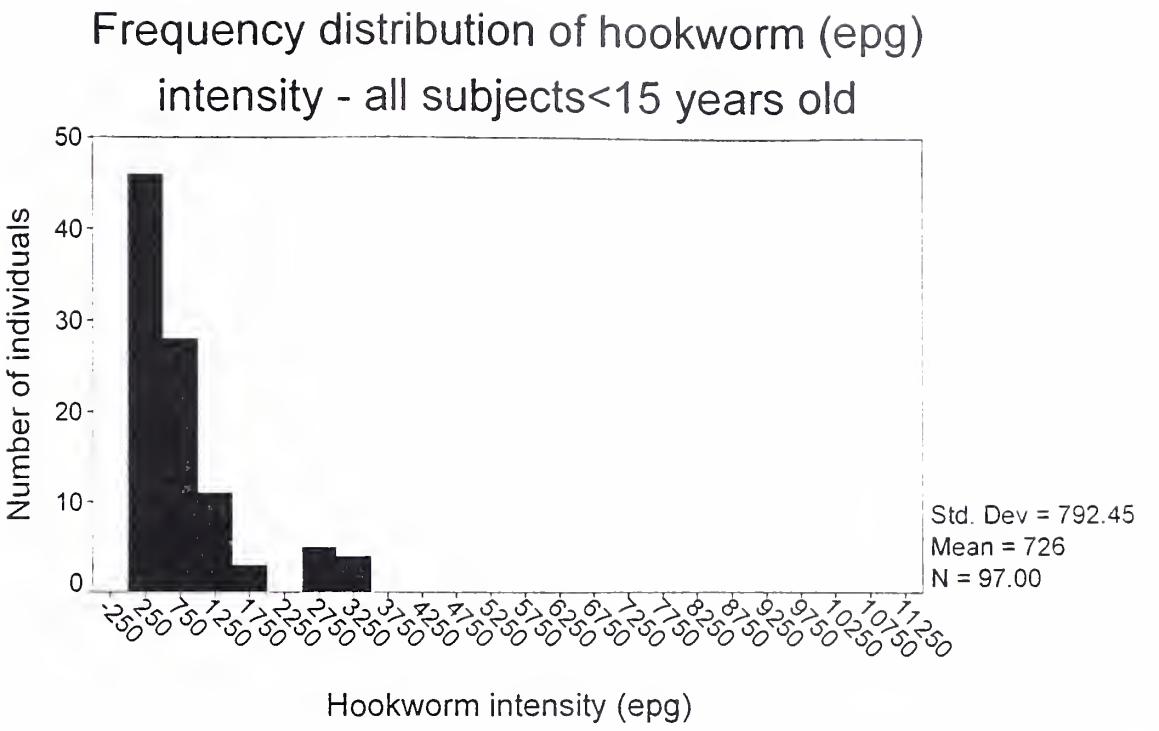
measured by eggs per gram stool). However, this overdispersion is highly age-dependent. Visual comparisons of hookworm distribution were made between the group of individuals less than 15 years old, and the group greater than 30 years of age (Figure 15). A shift to the right can be seen, indicating that low intensity infections are becoming relatively rarer and high intensity infections are becoming more common in older individuals. The degree of aggregation (at lower hookworm intensities) decreases significantly as the population ages, a phenomenon that will be discussed in future sections.

Table 4. Pilot study. Hookworm distribution (species unknown) among age groups. Intensity of infection is measured by eggs per gram feces. N=369 (those with hookworm data and age data)

	no infection	1-2499	2500-4999	5000-7500	Total
Age (yrs)					
0-2	99	9	-	-	108
3-5	24	9	-	-	33
6-10	41	12	2	-	55
11-15	17	12	-	-	29
16-20	4	10	-	-	14
21-25	10	20	2	1	33
26-30	9	23	2	-	34
31-35	13	14	4	2	33
36-40	7	10	1	-	18
41-45	6	2	-	-	8
46+	1	3	-	-	4
Total	231	124	11	3	369

Table 5. Summer study. *N. americanus* distribution among age groups. Intensity of infection is measured by eggs per gram feces. N=642 (those with hookworm data and age data)

	0	<1000	1000-2999	3000-4999	5000-9999	10000+	Total
Age (yrs)							
0-2	60	5	2	-	-	-	62
3-5	45	17	4	-	-	1	67
6-10	54	28	7	3	-	-	92
11-15	30	29	6	1	-	-	66
16-20	10	18	4	2	1	-	35
21-25	11	21	8	-	3	-	43
26-30	6	26	14	4	1	-	51
31-35	12	19	6	4	2	-	43
36-40	17	19	9	4	1	-	50
41-45	7	15	9	-	2	-	33
46-50	5	7	8	-	-	-	20
51-55	3	5	5	-	1	-	14
56-60	5	12	5	1	-	-	23
61-65	4	9	3	2	1	1	20
66+	5	9	3	1	-	-	18
Total	274 (42.7%)	239 (37.2%)	93 (14.5%)	22 (3.4%)	12 (1.9%)	2(0.3%)	642



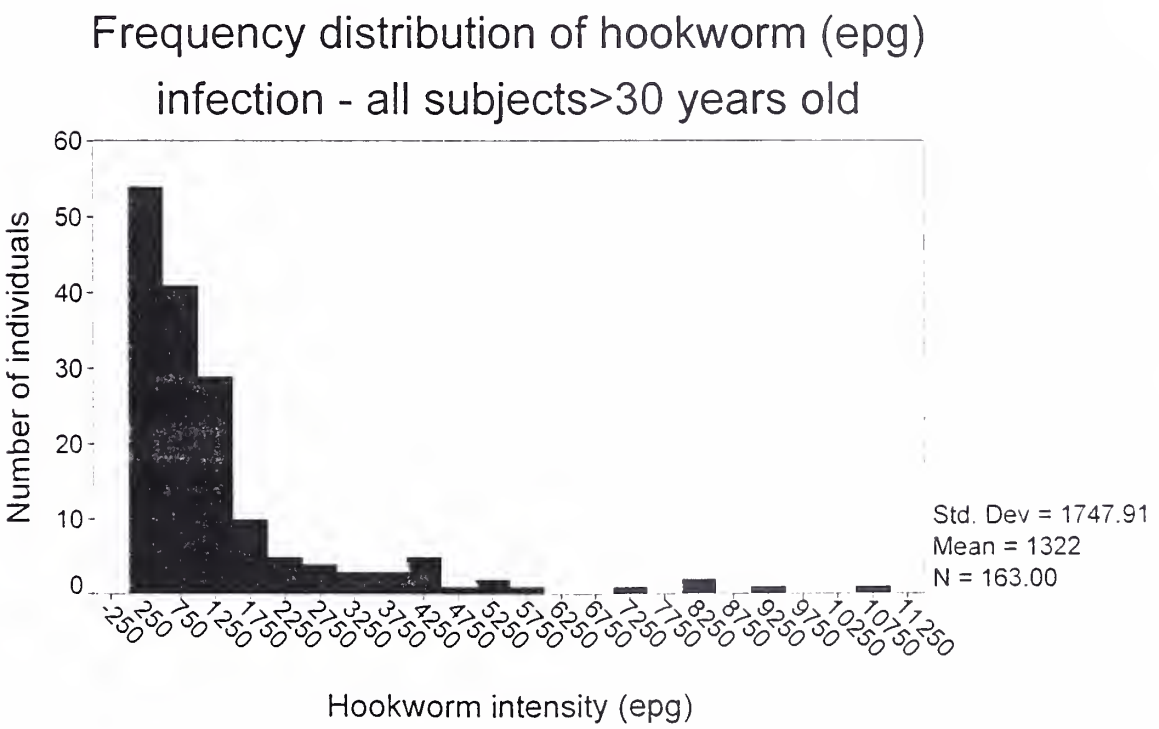


Figure 15. Summer study. Comparison of hookworm distribution between different age groups. The top graph represents all individuals with hookworm who are younger than 15 years of age, while the bottom graph represents all individuals with hookworm who older than 30 years of age. Notice the shift of hookworm intensity to the right in the bottom graph, indicating that low intensity infections are proportionally rarer and high intensity infections are more common. One case, a 3 year old female with 19,800 epg, was eliminated.

To perform comparisons of mean infection intensities between groups and to do other statistical analyses, these numbers were logarithmically transformed [log(hookworm epg +1)] to produce a normal gaussian distribution (Figure 16) for those with positive hookworm counts. All subsequent references to values of hookworm intensity will be measured as this transformation.

Hookworm distribution as a function of age:

Pilot study - Figure 17 shows the prevalence curve for hookworm infection (species unknown) across all age groups. Prevalence of infection is shown to be strongly age-related, with the accumulation of hookworm-infected people as age increases. Prevalence rates peak at approximately 70-75% at the 26-30 age group. It dips suddenly without explanation before rising again.

Table 6. Pilot study. Prevalence of mixed hookworm infection for all subjects across age classes. N=373

	Male		Female		Overall	
Age (yrs)	N	%	N	%	N	%
0-2	66	12	42	2	108	8
3-5	18	39	15	13	33	27
6-10	26	42	29	10	55	26
11-15	13	62	16	25	29	42
16-20	5	60	9	78	14	71
21-25	6	83	27	67	33	70
26-30	11	73	23	74	34	74
31-35	14	72	19	53	33	61
36-40	8	75	10	50	18	62
41-45	3	33	5	20	8	25
46+	4	75	-	-	4	75
Overall	199	35	174	40	373	37

The mean intensity of infection (as measured as eggs per gram stool and the log (hookworm epg + 1)) follows a very similar pattern to the prevalence curves. Low levels of hookworm infection are seen in early

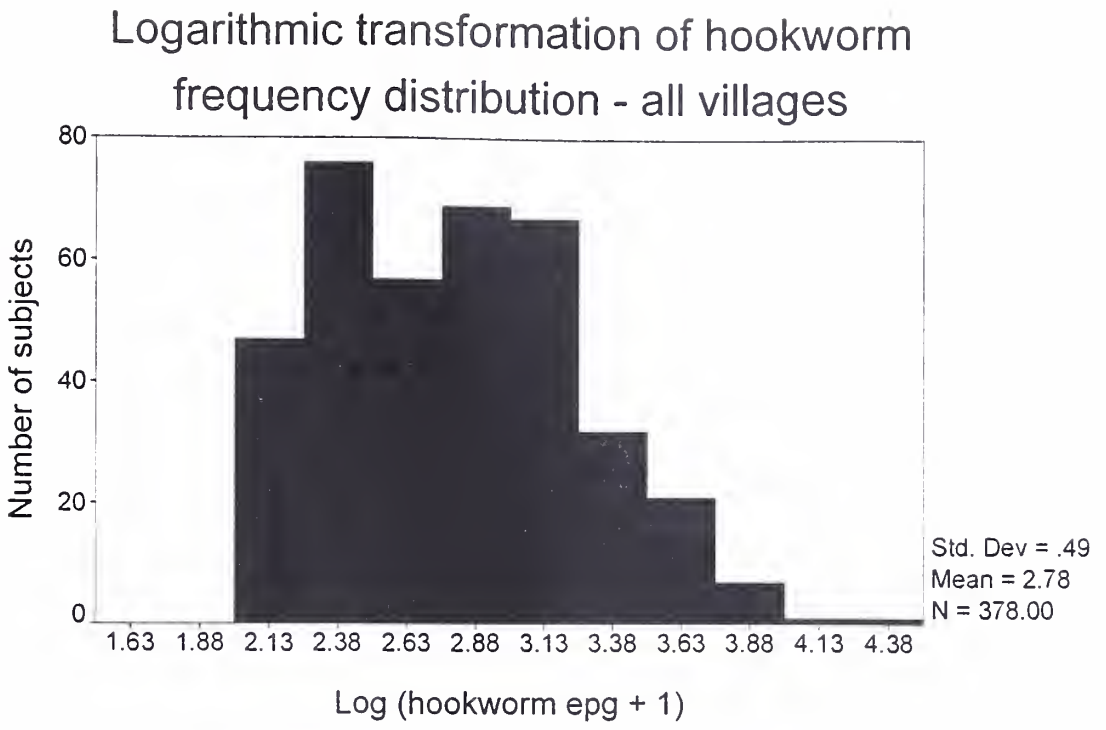


Figure 16. Summer study. Logarithmic transformation of hookworm intensities as measured by eggs per gram stool. Includes only those with *N. americanus* infections. The transformation (log (hkwm epg+1))was performed to obtain a more normal gaussian distribution for later comparisons of means and other statistical analyses. N=378

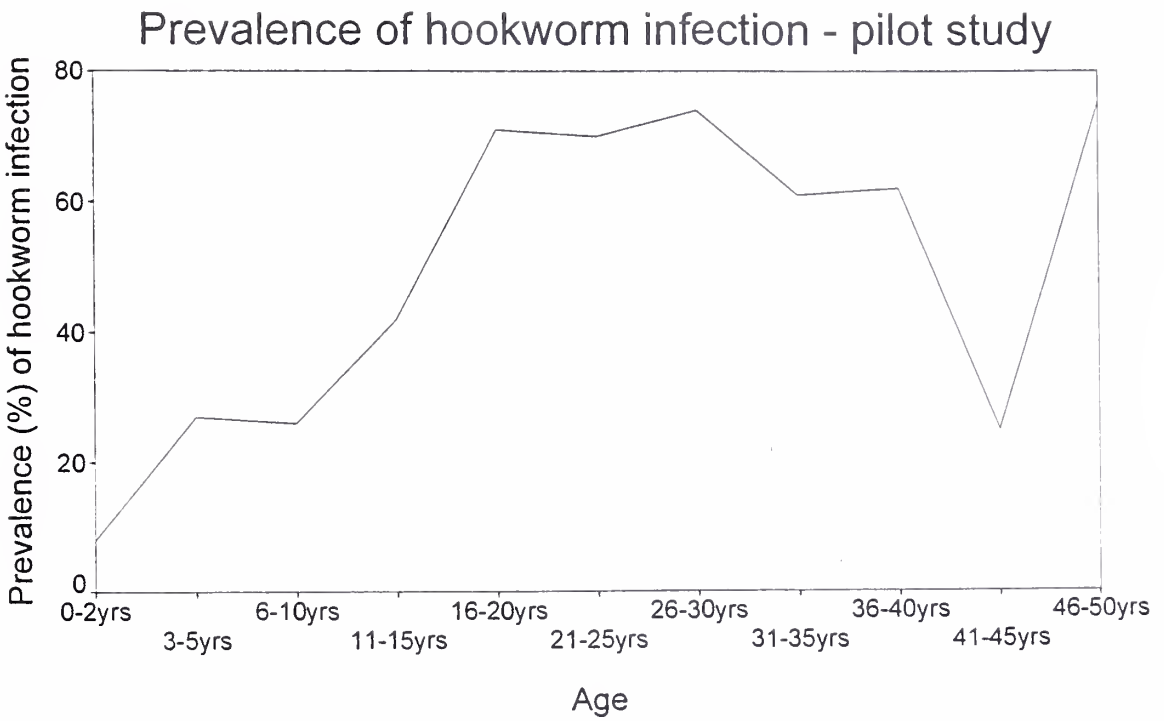
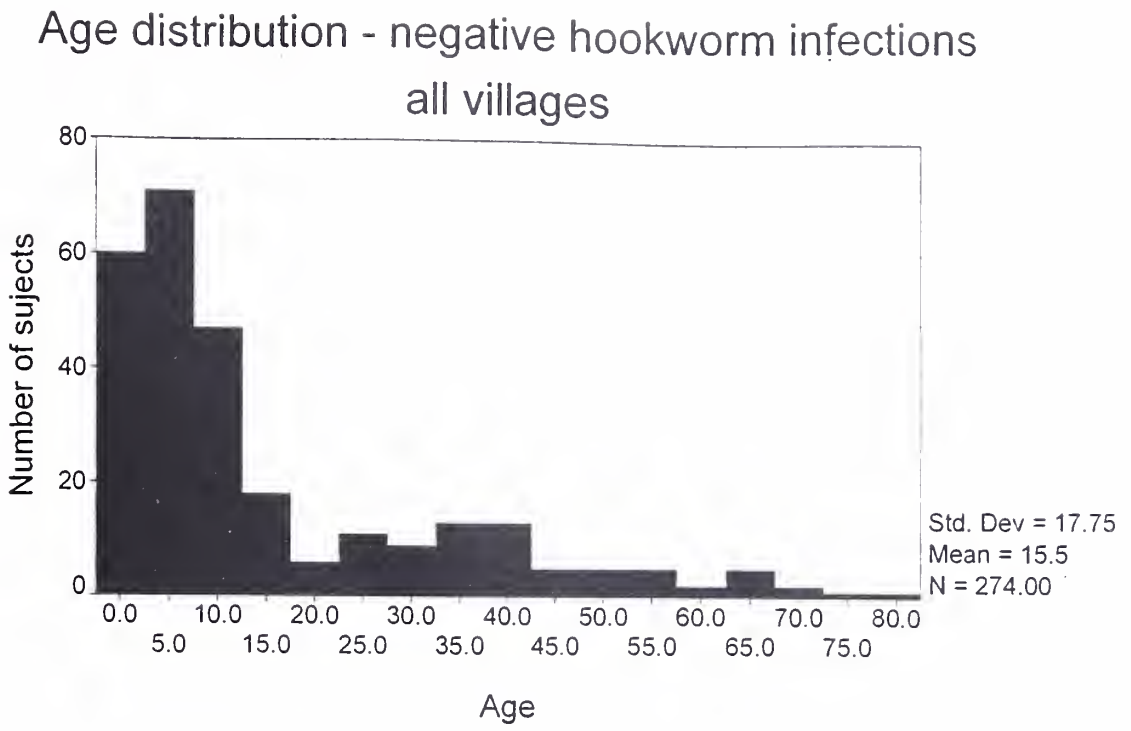


Figure 17. Pilot study. Age-prevalence profile of mixed hookworm infection for all subjects from the Nong Loo District of Sangkhlaburi. Prevalence of infection increases steadily until it peaks at the 26-30 year age group at approximately 75% prevalence. It drops markedly (and unexplainedly) in the 41-45 year old age group before rising again. N=373

childhood, with a linear rise in the intensity of infection through adolescence and adulthood, probably due to a rapid accumulation of worms and continual reinfection. The peak of infection levels occurs at the 31-35 year age group, a finding which is congruent with other studies showing a peak in early adulthood. Similar to the age-prevalence profile, the level of infection drops markedly in older adulthood, then returns. No explanation can be given for the sudden drop in intensity, though it may be caused by small sample size.

Summer study - the data included in this section includes both males and females across all age groups from all 3 villages. As compared to Figure 13, which is an age distribution of all those submitting a stool sample, the age distributions for those without *N. americanus* infections and those with infection have been shown in Figure 18. As expected, the cluster of individuals who are not infected with hookworm falls within the youngest age groups, and rapidly redistributes itself into the "infected" age profile as the subjects aged. From data taken from all three villages, Figure 19 shows that the overall prevalence of hookworm is age-dependent in the younger age groups, increasing linearly throughout childhood until prevalence peaks at approximately 80% of people infected. This peak occurs at age group 26-30. From this point, the prevalence of hookworm plateaus and remains constant throughout the older age groups without a rise or decline. Other studies have shown conflicting results - some with results similar to ours, and some finding a decrease in prevalence with increasing age.



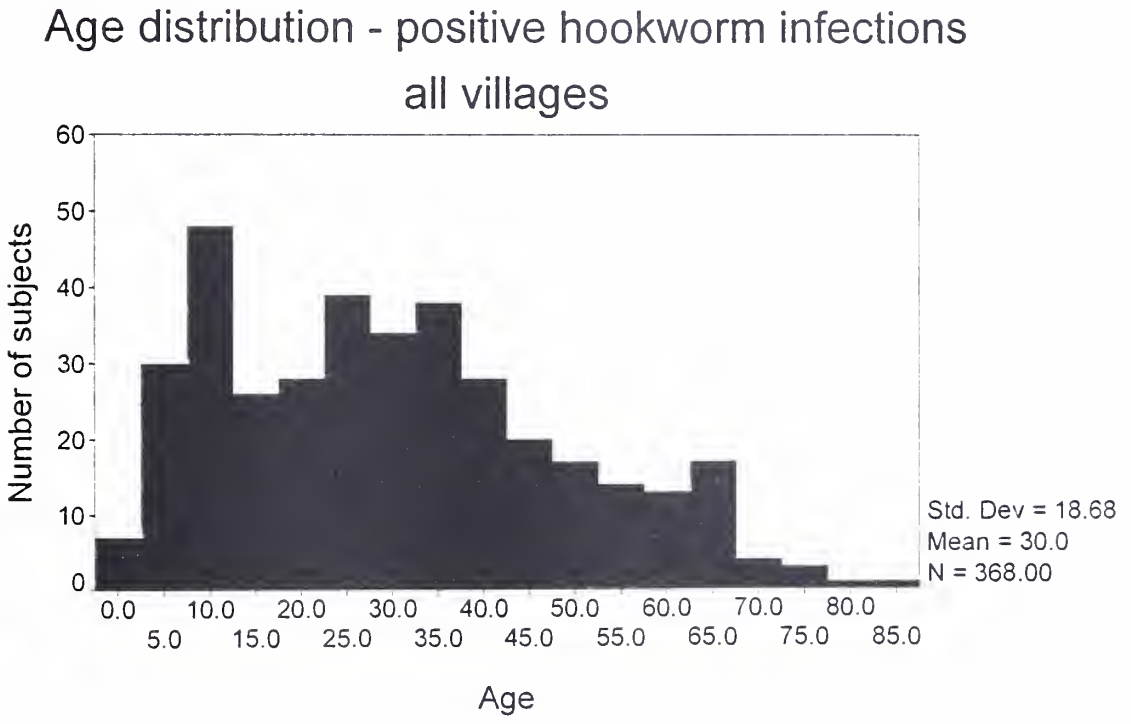


Figure 18. Summer study. Age distribution of those **without** *N. americanus infection* (N=282) on the top, and those **with** *N. americanus infection* (N=378) on the bottom. Note the rapid shift from the uninfected to the infected state starting in early childhood throughout early adulthood.

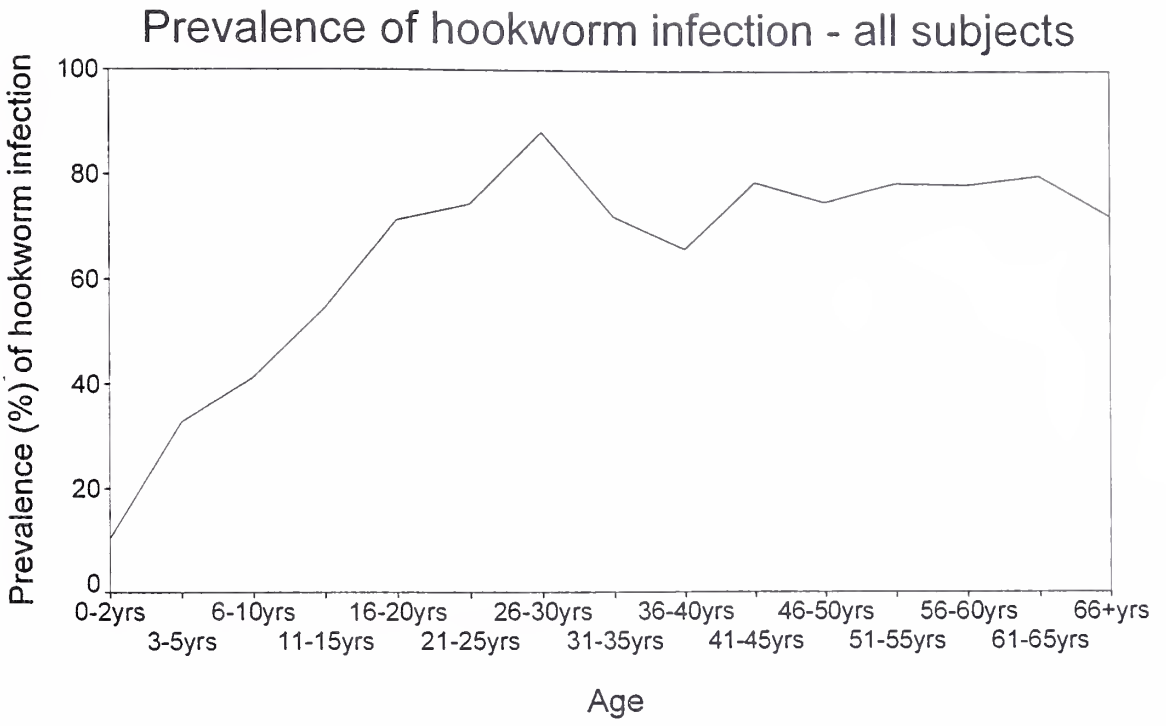


Figure 19. Summer study. Age-prevalence profile of *N. americanus* infection for all subjects submitting stool from all villages. Prevalence of infection increases in an almost linear fashion until the 26-30 year age group, then plateaus. We do not see either an increase or decrease in prevalence among older age groups. Includes all subjects with hookworm data. N=642

Table 7. Summer study. Prevalence of *N. americanus* for all subjects from all 3 villages, broken down by age classes. N=660

	Male		Female		Overall	
Age (yrs)	N	%	N	%	N	%
0-2	30	13.3	37	8.1	67	10.4
3-5	22	27.3	45	35.6	67	32.8
6-10	48	45.8	44	36.4	92	41.3
11-15	27	63.0	39	48.7	66	54.5
16-20	13	76.9	22	68.2	35	71.4
21-25	17	82.4	26	69.2	43	74.4
26-30	17	94.1	34	85.3	51	88.2
31-35	23	73.9	20	70.0	43	72.1
36-40	19	63.2	31	67.7	50	66.0
41-45	15	86.7	18	72.2	33	78.8
46-50	12	75.0	8	75.0	20	75.0
51-55	8	62.5	6	100.0	14	78.6
56-60	11	72.7	12	83.3	23	78.3
61-65	11	81.8	9	77.8	20	80.0
66+	9	66.7	9	77.8	18	72.2
missing age	11	72.7	7	28.6	18	55.6
Overall	293	60.1	367	55.0	660	57.3

The scatterplot of Figure 20 shows the natural distribution of hookworm intensity over the entire age range of our study. The mean intensities of infection (as they vary by age) mirror exactly the hookworm prevalence curves described above (Figure 21, top graph). The level of infection increases linearly through childhood, adolescence, and early adulthood until it peaks at the 26-30 year age group. Both prevalence and intensity dip slightly in the 36-40 year old age group before rising and plateauing at pre-dip levels. In our study population, *Necator americanus* seems to be an "adult disease" with a pattern of long-standing chronicity. No significant increase or decrease in hookworm intensity was seen with increasing age.

The mean intensity of infection was measured in two ways: using only those people infected with hookworm, and using all individuals regardless of their hookworm status. In the latter case, these mean intensity curves will incorporate prevalence rates into their curves (Figure 21, top graph), since the prevalence of hookworm is not constant throughout the entire age range.

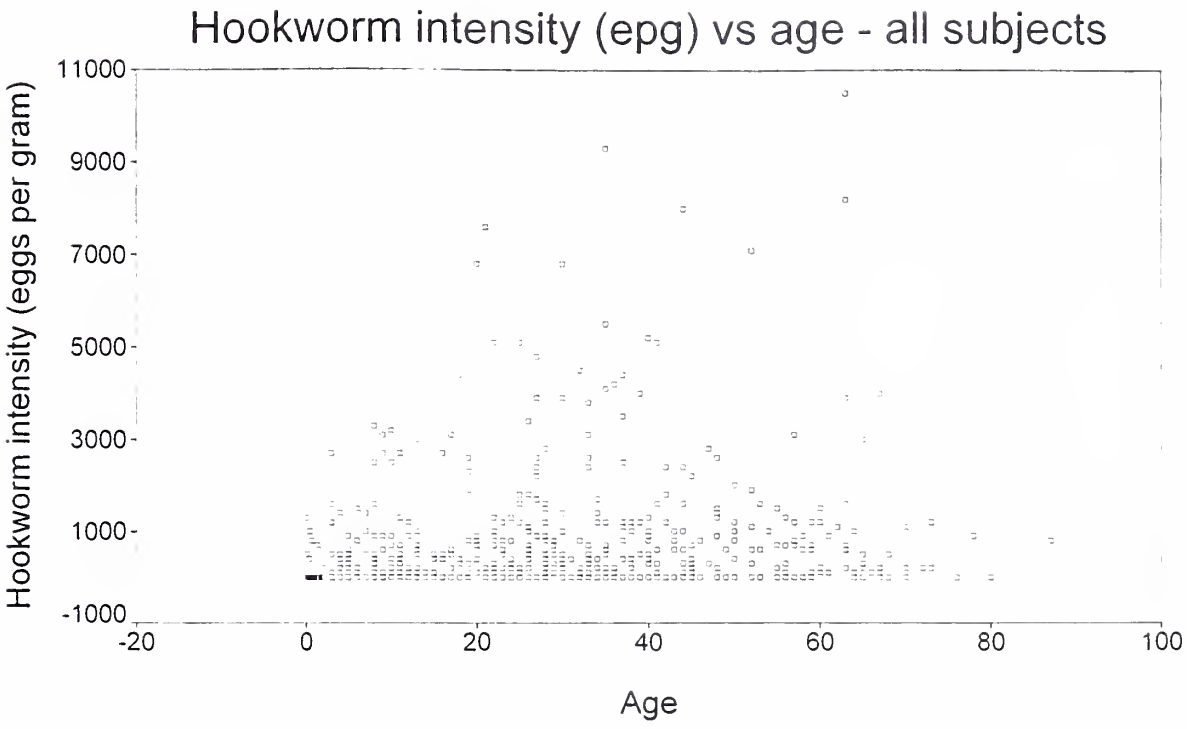
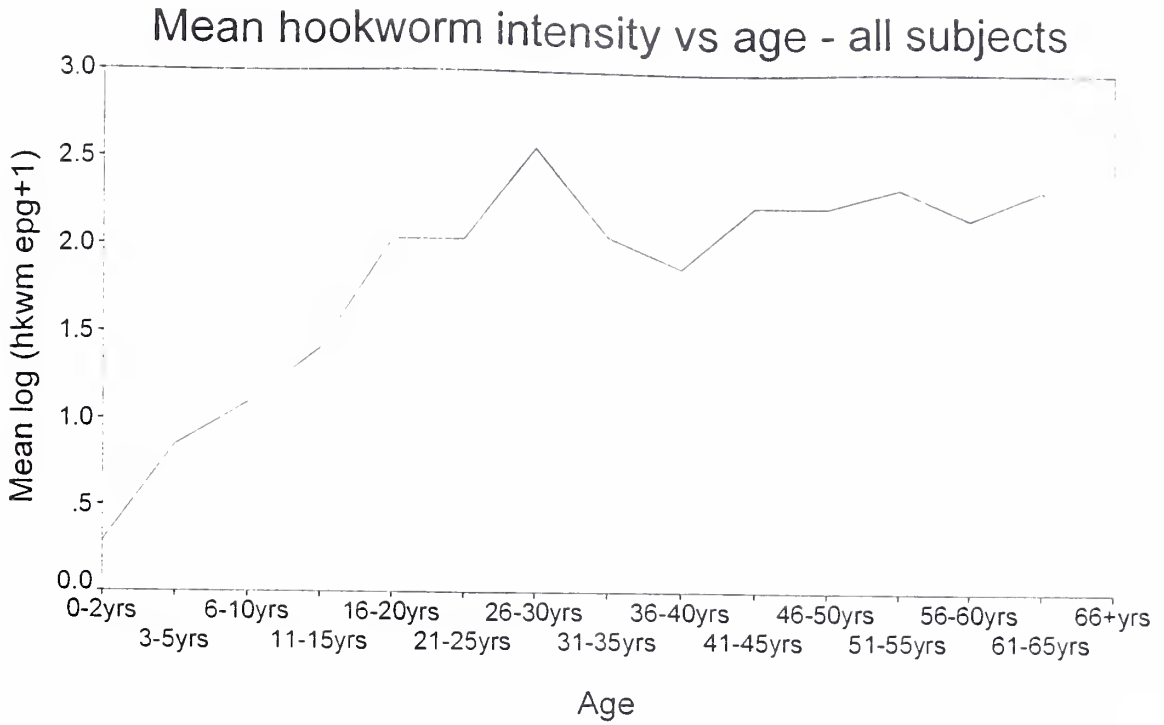


Figure 20. Summer study. Scatterplot of hookworm intensity vs age for all subjects submitting stool samples. Intensity is estimated by eggs per gram stool. One case, a 3 year old female with 19,800 epg, was eliminated. N=642



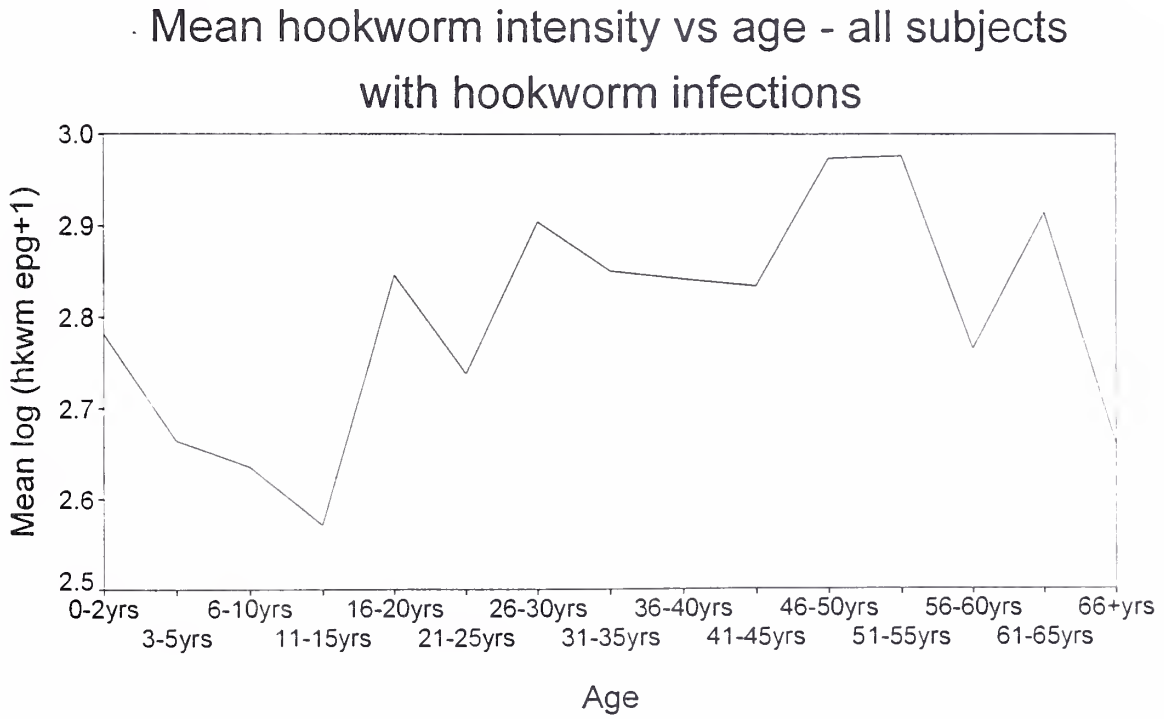


Figure 21. Summer study. Age-mean intensity profiles of *N. americanus* infection - all three villages included. Intensity of hookworm is estimated by eggs per gram stool. The top graph represents all subjects who submitted stool samples (regardless of hookworm infection status), and therefore takes into account prevalence as well (N=642). The bottom graph includes only those with positive hookworm egg counts (N=378). From both curves, one subject (a 3 year old female with 19,800 epg) was eliminated.

The prevalence of hookworm is much lower in early childhood, implying that more "0 epg"'s will be factored into the measurement of mean intensity at the younger age groups. Therefore, the mean intensities of infection will be "skewed" towards lower infection levels. The former way of measuring mean intensity is good for looking at how hookworm burden varies in individuals with increasing age (Figure 21, bottom graph). The latter way of measuring mean intensity is valid for examining the variation in *overall* levels of infection between entire age groups, and takes hookworm prevalence into account. For the remainder of the Results section, the measurement of infection intensity will imply only those who have positive hookworm egg counts.

Hookworm distribution as a function of village:

During the summer study, there were 3 villages studied which had strikingly different socioeconomic conditions and ethnic make-up. As stated earlier, the inhabitants of Villages 1 and 3 were predominant of Mon descent, while those in Village 2 were Karen. Overall prevalence of *Necator* was 61.2%, 45.9%, and 60.7% for Village 1, 2, and 3 respectively. Figure 22 and Table 8 compare the prevalence curves (as a function of age) between the 3 villages. It is easy to discern that Village 2 is not infected to the same degree as Villages 1 and 3, though Villages 1 and 3 are very similar in both the levels and age distribution of hookworm infection. Villages 1 and 3 show convexity in their prevalence curves, with a slight decrease in hookworm prevalence with increasing age.

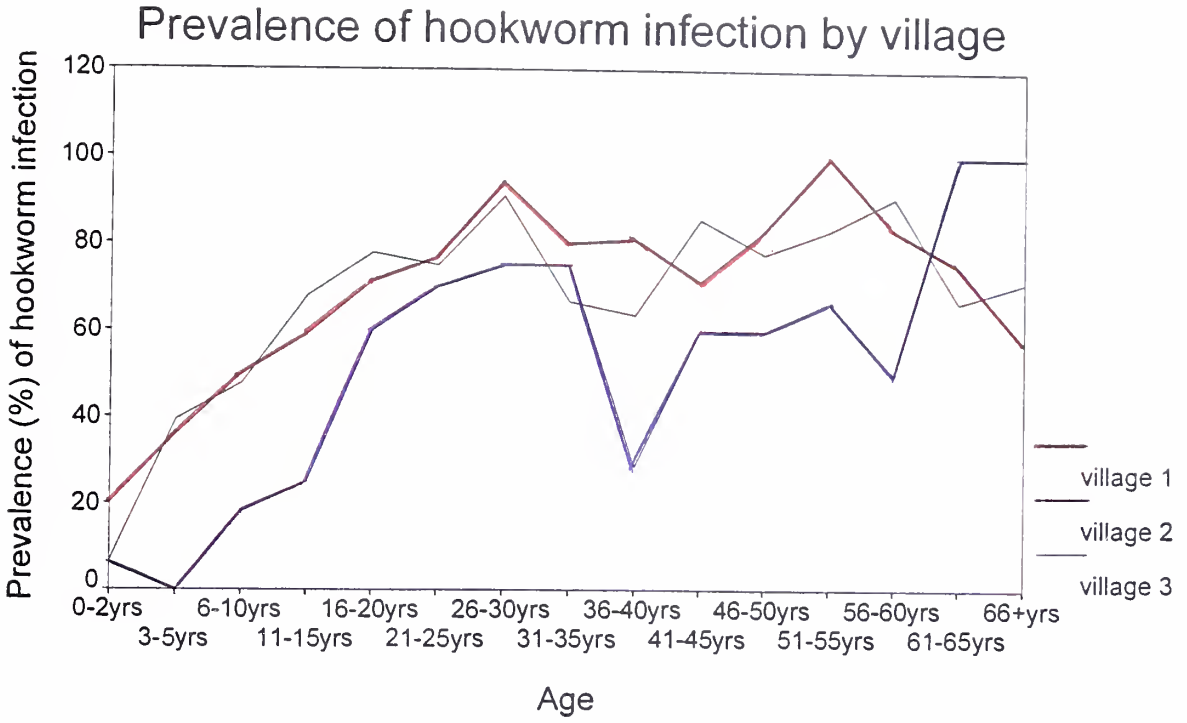


Figure 22. Summer study. Comparing the age-prevalence profiles of *Necator americanus* infection between the three villages. All subjects submitting stool were included. Village 2 consistently has a lower prevalence of *N. americanus* than Villages 1 and 3, which have similar prevalence levels. As with the overall population, there is an almost linear increase in prevalence until 26-30 years of age, when it then plateaus. We do not see either an increase or decrease in prevalence among older age groups. N=193 Village 1, N=147 Village 2, N=302 Village 3, N=642 total

Table 8. Summer study. Prevalence of *Necator americanus* infection by village for all subjects with hookworm data. Stratified into age groups.

	Village 1		Village 2		Village 3	
Age (yrs)	N	%	N	%	N	%
0-2	20	20.0	16	6.3	31	6.5
3-5	25	36.0	9	0.0	33	39.4
6-10	26	50.0	22	18.2	44	47.7
11-15	22	59.1	16	25.0	28	67.9
16-20	7	71.4	10	60.0	18	77.8
21-25	13	76.9	10	70.0	20	75.0
26-30	17	94.1	12	75.0	22	90.9
31-35	10	80.0	12	75.0	21	66.7
36-40	21	81.0	7	28.6	22	63.6
41-45	7	71.4	5	60.0	21	85.7
46-50	6	83.3	5	60.0	9	77.8
51-55	2	100.0	6	66.7	6	83.3
56-60	6	83.3	6	50.0	11	90.9
61-65	4	75.0	7	100.0	9	66.7
66+	7	57.1	4	100.0	7	71.4
missing age	3	0.0	12	0.0	3	66.7
Overall	196	61.2	159	45.9	305	60.7

The Pearson chi-square test comparing the prevalences of *N. americanus* in these 3 villages concluded that the differences in prevalence were too great for the samples to "be from the same population" (P=0.004), though Villages 1 and 3 seem to have similar host characteristics that make their hookworm distribution more identical. The risk of a person being infected with hookworm in Village 2 was about 55% (odds ratio=0.55, 95% CI of 0.37-0.79) of the risk of being infected within the rest of the studied population. Likewise, the odds ratio for Village 1 was 1.98 (95% CI of 1.39-2.82) - almost twice the risk compared to the remainder of the population. For Village 3, the odds ratio was 0.77 with a 95% CI of 0.56-1.07.

Figures 23 and 24 show visually the differences in intensity between the 3 villages. The highest levels of infection were found in Village 1, then in Village 3 , then Village 2. Considering that the 95% confidence intervals show very little overlap between Village 2 and the other villages, the difference in the mean intensities of infection is real. ANOVA of log (hookworm epg + 1) by village likewise showed that there were significant

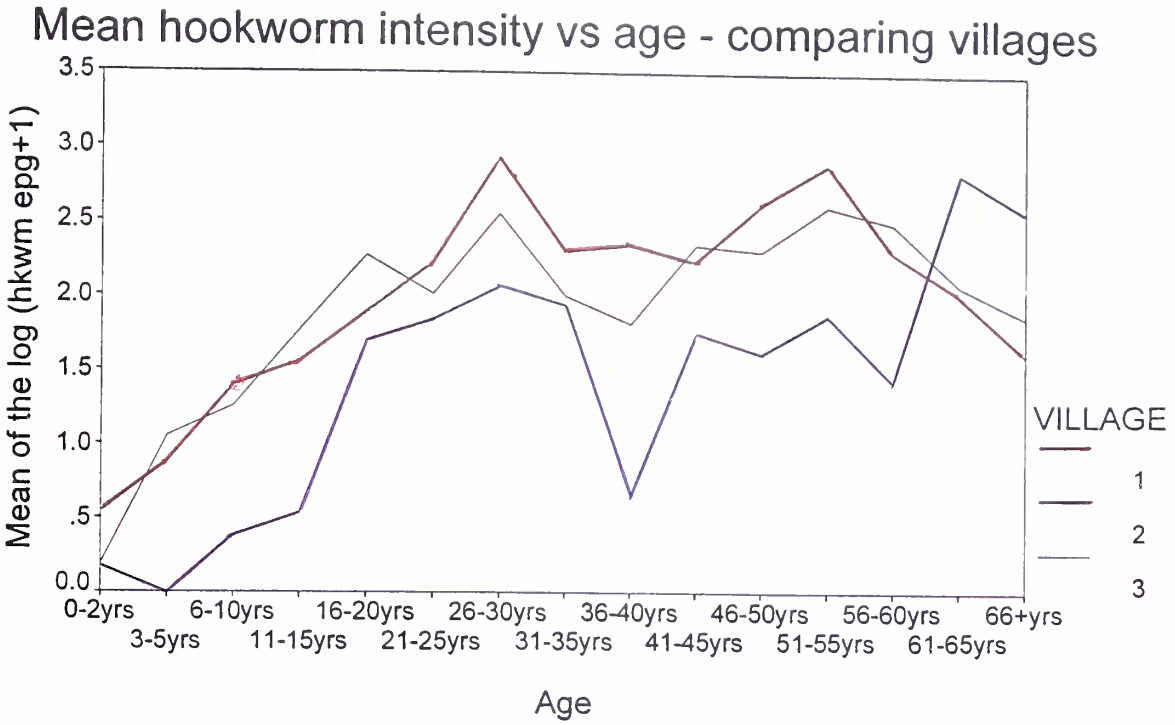


Figure 23. Summer study. Comparing the age-mean intensity profiles for *N. americanus* infections between the three villages. All subjects submitting a stool sample were included, regardless of hookworm infection status. Intensity is estimated by eggs per gram stool. The age-intensity profiles mirror the age-prevalence curves. Village 2 consistently has a lower mean level of infection, while that of Villages 1 and 3 are comparable. One subject, a 3 year old with 19,800 epg, was eliminated. N=641

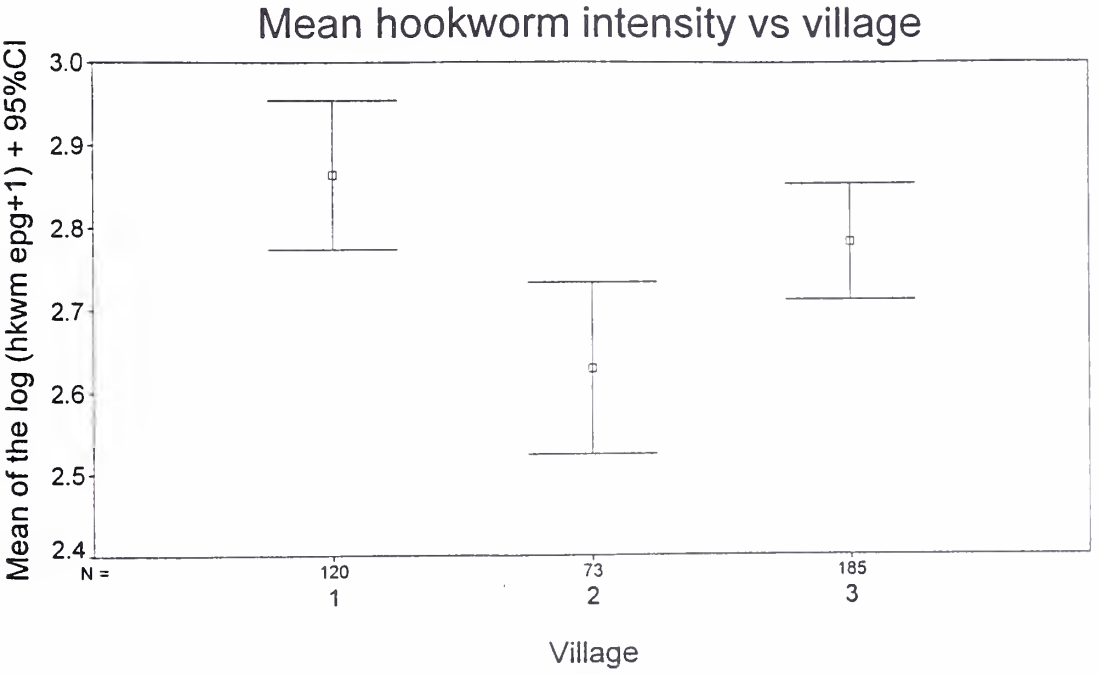


Figure 24. Summer study. Plots comparing the overall mean intensity of *N. americanus* infections between the three villages. Only those with positive hookworm egg counts were included. Intensity is estimated by eggs per gram stool. Highest mean intensity was found in Village 1, then Village 3, then Village 2. N=642

differences between the 3 villages in terms of intensity of infection (F ratio = 5.3841, *P*=0.005).

T-tests of intensity of hookworm infection between pairs of villages showed accordant results. Villages 1 and 2 as well as Villages 2 and 3 showed statistically significant differences in the level of hookworm infection in those subjects positive for hookworm infection (Table 9). On the other hand, in Villages 1 and 3 (both predominantly Mon-inhabited villages) the difference in infection intensity was insignificant. Therefore, the Karen village (Village 2) distinguishes itself from the other two villages in that it has a much lower overall level of hookworm infection.

Table 9. Summer study. T-test results comparing the mean intensity of *Necator americanus* infection between the villages. The Karen village (Village 2) distinguishes itself from the other two villages in having a lower level of hookworm infection. On the other hand, Village 1 and 3, both the predominantly Mon-inhabited villages, the differences in infection intensity were insignificant.

Village	Levene's Test for Equality of Variances		T-tests for Equality of Means	
	F	*P*	Equal *P*	Unequal *P*
1 and 2	0.873	0.351	0.001	0.001
2 and 3	0.334	0.564	0.020	0.017
1 and 3	0.222	0.683	0.157	0.159

The Tukey (HSD) Pairwise Comparisons of Means test affirms the above results, showing that the level of infection for Village 1 and 3 were not significantly different from one another (Critical Q value - 3.314), but that Village 2 was different from the other two.

Hookworm distribution as a function of sex:

Summer study - both male and female groups were analyzed separately to look for possible differences in the distribution of hookworm. For both sexes, the prevalence of hookworm infection across age groups closely followed the earlier patterns shown for the overall population (Table 7 and

Figure 19). Prevalence of hookworm infection between males and females (60.1% vs 55%) did not seem to differ overall, as demonstrated by the Pearson chi-square test comparing prevalence of hookworm infection in males versus females (P = 0.1946). Likewise, there was no significant increase in risk for either sex for being infected with hookworm.

Within each age group, comparisons of prevalence in males vs females gave P values which were all greater than 0.25, except for the 51-55 age group (P = 0.09) though the sample size was small for this group (N=14) (Table 7). These results indicate that the prevalence of *Necator* infection did not differ significantly between males and females overall and within each age group, and that sex itself is not a factor affecting the distribution of infection.

On first glance, the prevalence of infection in males appears to be consistently higher than that of females for the 6-30 year old age range (Figure 25). However, on further investigation of the subjects 6-30 years old, the Pearson chi-square test showed the difference between males and females to be insignificant (P = 0.305), and that the risk of infection was not significantly increased in males (odds ratio=1.288, 95% CI of 0.77-2.15) in this age range. Although not statistically significant, the higher prevalence in males may be occupationally-related or some other unknown factor.

Likewise, the prevalence of infection in females seems generally higher that that of males in many of the older age groups (50 years of age and beyond). However, statistical analysis showed no notable discrepancies between the two sexes. One theory which has been proposed is the possible positive selection pressure for women. Women generally tend to live longer than men, and it is possible that men with hookworm disease have more of a tendency to die, leaving only those men without hookworm infection.

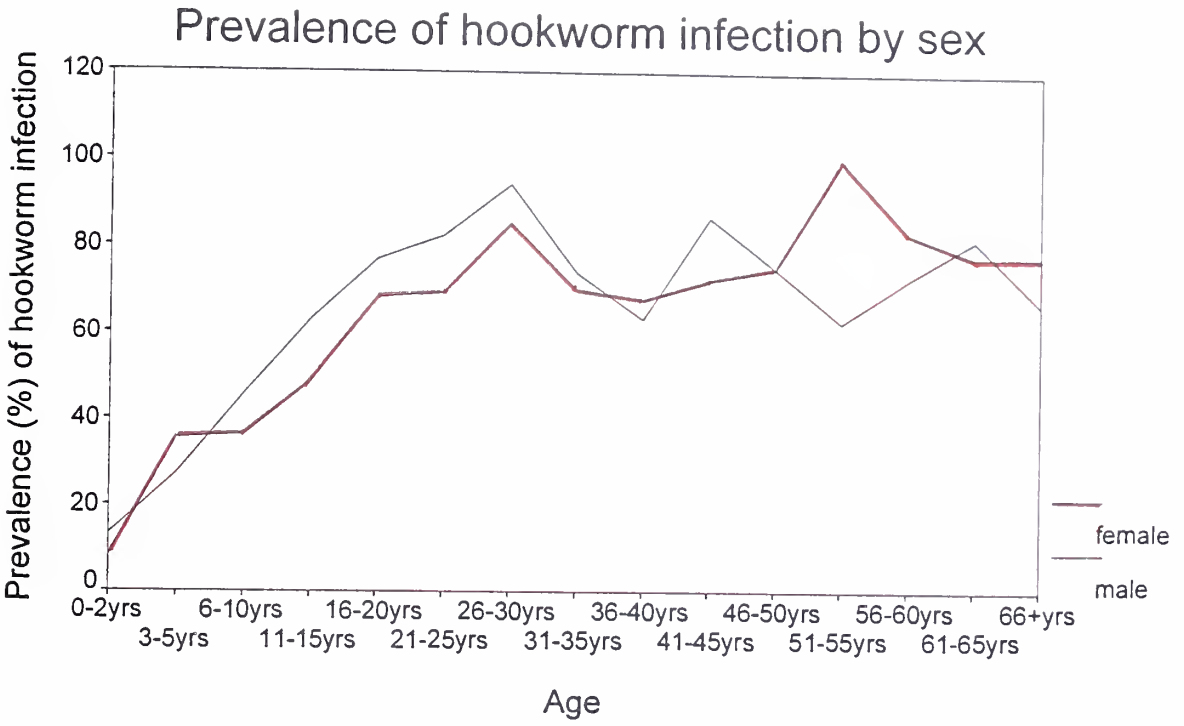


Figure 25. Summer study. Comparing the age-prevalence profiles of *Necator americanus* for males and females. All subjects who submitted stool were included. Both sexes increase linearly until age group 26-30, when the prevalence reaches a plateau which is more or less sustained throughout later adulthood. Males seem to have a slightly higher prevalence of infection between the ages of 6-30, but it is not statistically significant. N=642

Also, in the small group of people missing an age value, prevalence was 72.7% for males compared to only 28.6% for females. However, the sample size was small (N=18) and the Pearson chi-square test found only borderline significance ($P = 0.066$).

In comparing mean intensities of infection between the two sexes, the plots in Figures 26 and 27 visually show some evidence of sex-dependent differences (females with a slightly higher mean level of infection), but the t-tests comparing these means deny any significant difference. The t-tests for the individual age groups (Table 10) do not reveal any difference in the level of infection between the sexes, with most P values >0.10. Because the prevalence of hookworm was consistently higher in males in the 6-30 age range (though not statistically significant), comparisons were also made with the intensity of infection.

Table 10. Summer study. T-test comparing levels of hookworm infection between males and females, overall and within age groups.

	Levene's Test for Equality of Variances		T-tests for Equality of Means	
	F	*P*	Equal *P*	Unequal *P*
all ages	0.000	0.998	0.549	0.549
6-30 years old	0.990	0.321	0.259	0.258
0-2	1.186	0.325	0.426	0.398
3-5	1.659	0.212	0.103	0.229
6-10	0.196	0.661	0.574	0.572
11-15	0.191	0.664	0.765	0.766
16-20	0.839	0.369	0.091	0.101
21-25	0.272	0.606	0.436	0.439
26-30	0.115	0.736	0.372	0.385
31-35	6.438	0.017	0.242	0.263
36-40	0.233	0.633	0.438	0.470
41-45	0.550	0.465	0.264	0.264
46-50	0.616	0.447	0.652	0.684
51-55	0.169	0.691	0.436	0.425
56-60	3.654	0.074	0.670	0.688
61-65	1.871	0.193	0.985	0.986
66+	0.013	0.910	0.066	0.064

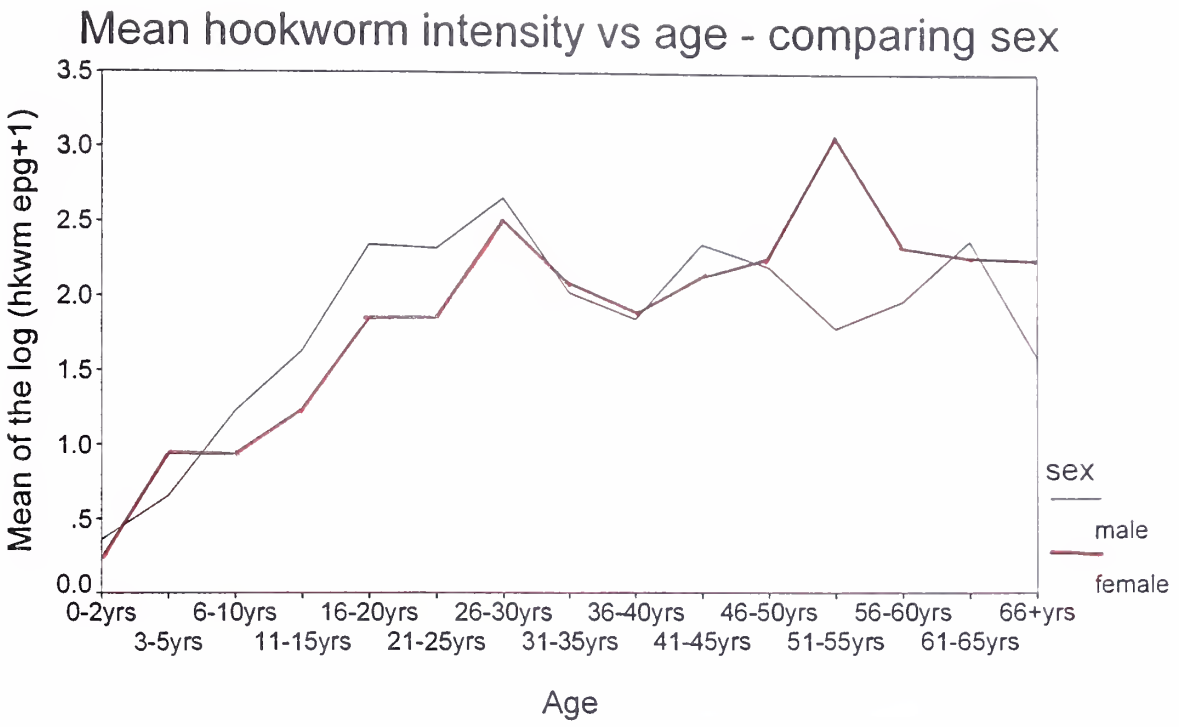


Figure 26. Summer study. Comparing the age-mean intensity profiles of *N. americanus* infections for males and females. All subjects who submitted a stool sample were included, regardless of hookworm status. Intensity is estimated by eggs per gram stool. The age-intensity profiles mirror the age-prevalence curves, in which males seem to have a slightly higher level of infection between the ages of 6-30, but it is not statistically significant. One subject, a 3 year old with 19,800 epg, was eliminated. N=641

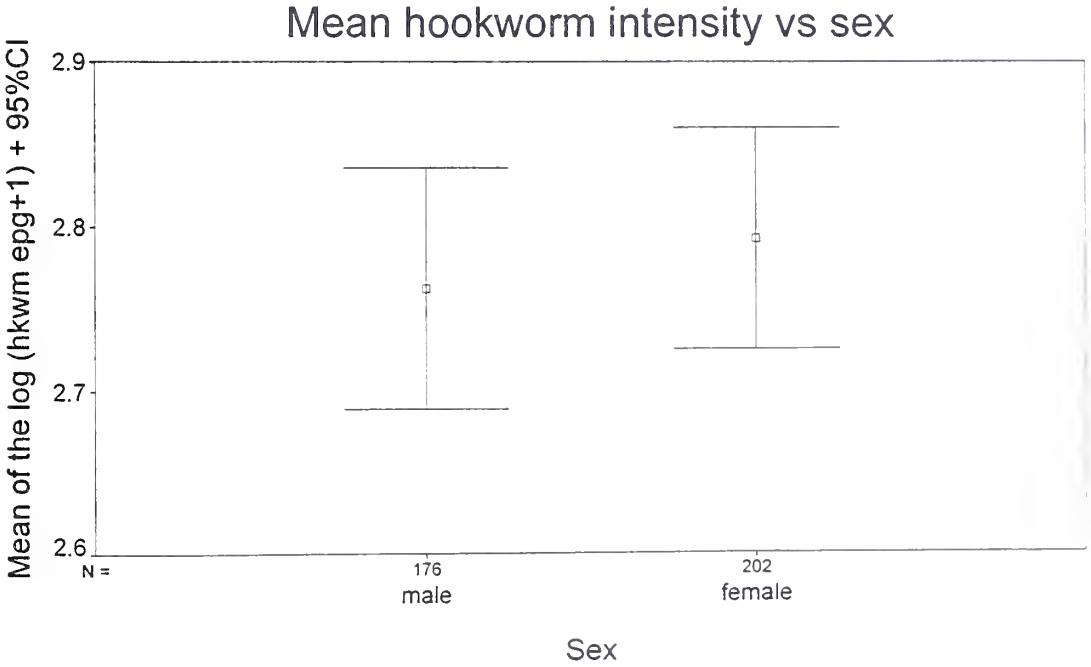


Figure 27. Summer study. Plots comparing overall mean intensity of *N. americanus* infection (as measured by eggs per gram stool) between males and females. Only those with positive hookworm egg counts were included. There seems to be no significant difference in the mean hookworm intensity between the two sexes. N=378

Ascaris distribution:

Of those subjects submitting stool samples (N=660), only 42 samples were positive for *Ascaris* infection. Like hookworm, the intensity of *Ascaris* infections follows that of a negative binomial distribution (Figure 28), in which most cases are light infections (<10000 eggs per gram feces), but with 16% of the people producing 62.7% of the *Ascaris* eggs.

For statistical purposes, these numbers were also transformed logarithmically [log(*Ascaris* epg +1)] in an attempt to produce a more normal frequency distribution. All subsequent references to values of *Ascaris* intensity will be measured as this transformation.

Age- and sex-dependence of Ascaris infection:

Table 11. Pilot study. Prevalence of *Ascaris lumbricoides* infection for all subjects with *Ascaris* data from all villages. Prevalence% calculated from those with known ages.

	Male		Female		Overall	
Age (yrs)	N	%	N	%	N	%
0-2	66	13	42	14	108	14
3-5	18	28	15	20	33	24
6-10	26	27	29	10	55	18
11-15	13	8	16	25	29	17
16-20	5	20	9	11	14	14
21-25	6	17	27	15	33	15
26-30	11	-	23	13	34	9
31-35	14	-	19	2	33	6
36-40	8	13	10	1	18	11
41-45	3	-	5	-	8	-
Overall	174	15	199	14	373	14

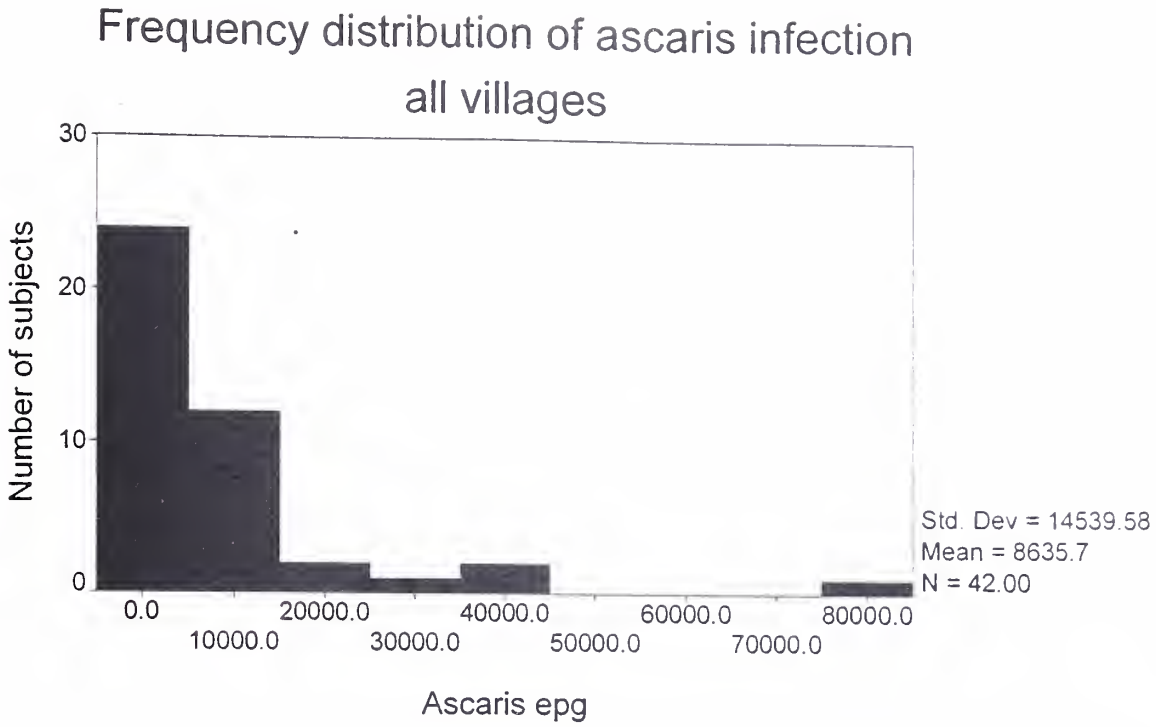


Figure 28. Summer study. *Ascaris lumbricoides* intensity (eggs per gram stool) histogram of all subjects with positive *Ascaris* egg counts. Like hookworm, the histogram also shows a negative binomial distribution. N=42

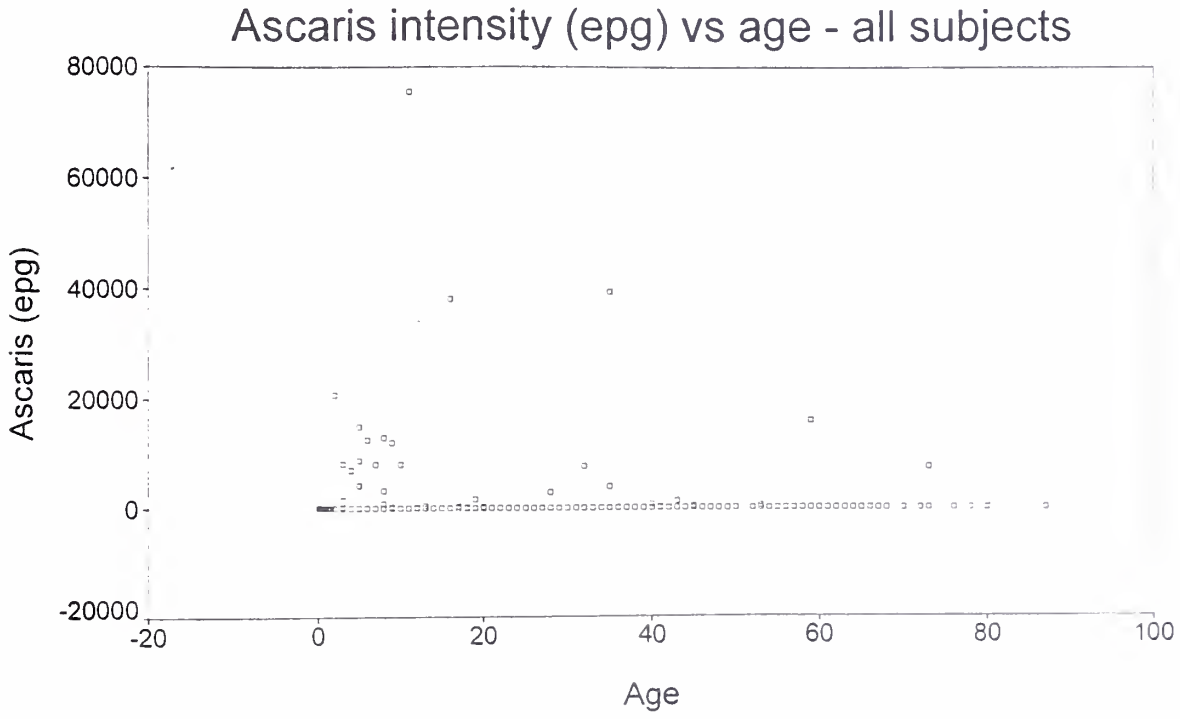


Figure 29. Summer study. Scatterplot of *Ascaris* intensity vs age for all subjects submitting stool samples. Intensity is estimated by eggs per gram stool. N=642

Table 12. Summer study. Prevalence of *Ascaris lumbricoides* infection for all subjects with *Ascaris* data from all villages. Prevalence% calculated from those with known.

	Male		Female		Overall	
Age (yrs)	N	%	N	%	N	%
0-2	30	-	37	5.4	67	2.9
3-5	22	4.5	45	15.6	67	11.9
6-10	48	12.5	44	9.1	92	10.9
11-15	27	7.4	39	7.7	66	7.6
16-20	13	7.7	22	18.2	35	14.3
21-25	17	-	26	-	43	-
26-30	17	-	34	2.9	51	2.0
31-35	23	4.3	20	10.0	43	7.0
36-40	19	-	31	3.2	50	2.0
41-45	15	13.3	18	5.6	33	9.1
46-50	12	-	8	-	20	-
51-55	8	12.5	6	-	14	7.1
56-60	11	9.1	12	-	23	4.3
61-65	11	-	9	-	20	-
66+	9	-	9	11.1	18	5.6
missing age	11	-	7	14.3	18	5.6
Overall	293	5.1	367	7.4	660	6.4

Pilot study - Peak *Ascaris* prevalence fell in the 3-5 year age group, which is consistent with other studies and is to be expected. Prevalence peaked at 24% in this age group, then decreased rapidly (Table 11).

Summer study - This data includes both males and females from all 3 villages. The scatterplot of Figure 29 show naturally the distribution of *Ascaris* over the age range. As is often the case with *Ascaris*, the majority of the infections cluster in the early ages of childhood until the approximate age of 10 or 15, when the prevalence of *Ascaris* begins to drop off. Table 12 and Figure 30 show this result - the highest prevalence at early childhood with a rapid drop-off and low levels through adulthood. There are no discernible trends during adulthood, probably due to the small number of people actually infected with ascariasis.

The presence of *Ascaris* during the summer seems to be considerably less than that in the population studied during the pilot study. Peak prevalence reached only 14%, and only 12% in the 3-5 year age group. Unlike

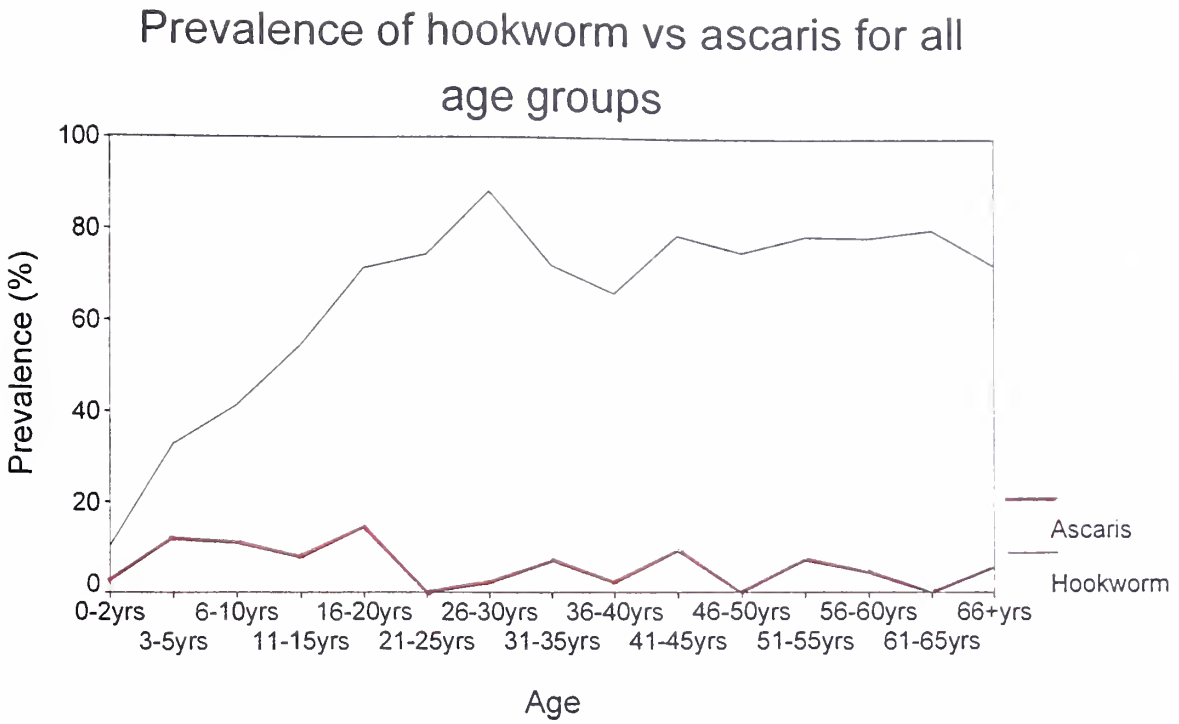


Figure 30. Summer study. Comparing age-prevalence profiles for *N. americanus* and *Ascaris lumbricoides*. All subjects submitting a stool sample were included. N=642

the January study, when overall *Ascaris* prevalence was 14%, the summer study gave an overall *Ascaris* prevalence of 6.4%.

Compared to the prevalence for hookworm infection in these populations, the overall prevalence of ascariasis was considerably smaller in both the January and the summer studies (Figure 30 from the summer study).

There seemed to be no association between sex and prevalence of infection, and there was no significant difference in the prevalence of *Ascaris* in males versus females. 5.1% of males and 7.4% of females who had turned in stool samples were *Ascaris*-infected (Pearson chi-square $P = 0.242$).

Since the number of people positive for *Ascaris* infections numbered only 42 for the entire study, it was impossible to do analyses to compare the mean levels of infection or to break the sample into age groups. The limited number of data points would make such comparisons meaningless and inaccurate.

Co-infection with hookworm and Ascaris:

In many populations worldwide, infection with one geohelminth implies possible co-infection with other geohelminths. *Ascaris*, *Trichuris*, and hookworm are often found co-inhabiting its hosts, either in pairs or all three. However, it is very rare to find populations with pure hookworm infections. Of the 660 people submitting stool samples (summer study), 356 had pure hookworm infections, 20 had pure *Ascaris* infections, and 22 were positive for both (Table 13). In other words, only 5.8% of those infected with hookworm were co-infected with *Ascaris*. Compared to many other studies of rural areas, there is very little co-infection here by *Ascaris* or *Trichuris* (*Trichuris* data unavailable).

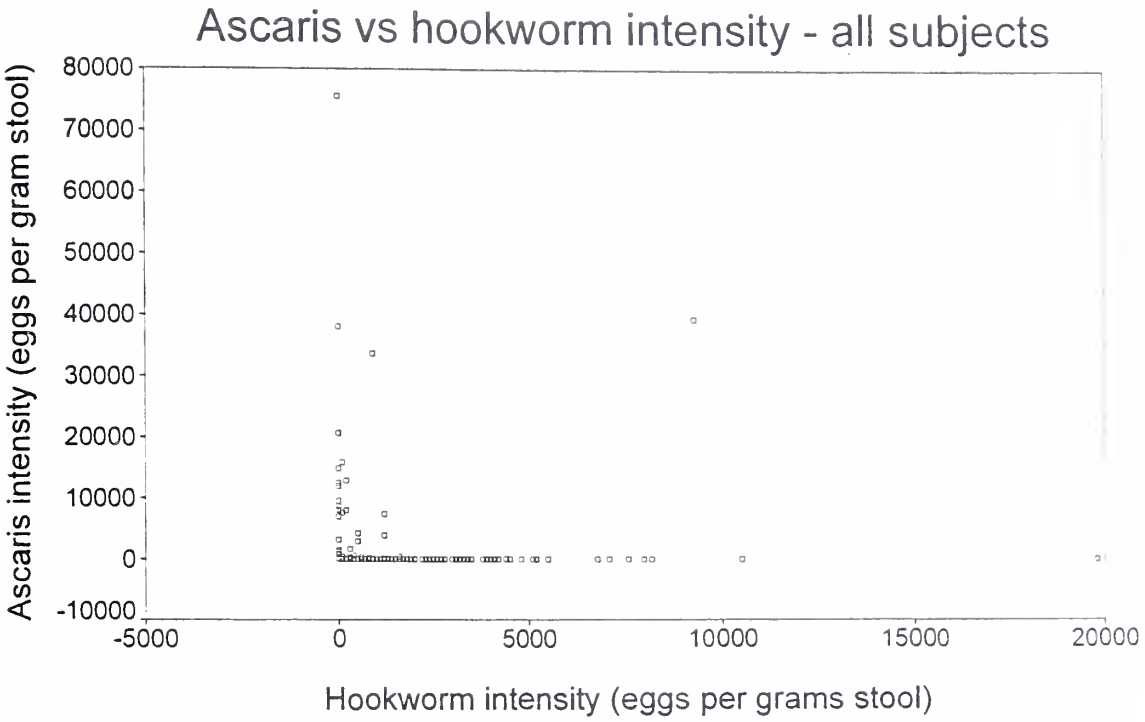


Figure 31. Summer study. Scatterplot of *Ascaris* intensity (in eggs per gram stool) vs *N. americanus* intensity (in eggs per gram stool) to look at the relationship between their levels of infections. The plot shows a paucity of dually-infected individuals, with most subjects having pure *Necator*.

		Ascaris +	*Ascaris* -
Necator	+	22	356
	-	20	262

Table 13. Summer study. Co-infection between *Ascaris* and *N. americanus*. Of the 660 people submitting stool samples, 356 had pure hookworm infections, 20 had pure *Ascaris* infections, and 22 were dually infected. Only 5.8% of those infected with hookworm were co-infected with *Ascaris*. Pearson chi square P=0.508, indicating that there is no association between hookworm infection status and *Ascaris* infection status.

The Pearson chi-square test showed no relatedness between having hookworm and having *Ascaris* (P=0.508): that those without hookworm were just as likely to have *Ascaris* as those with hookworm. Those with hookworm infections were not at either increased or decreased risk for *Ascaris* infections (odds ratio = 0.810, 95% CI of 0.41-1.58) than those without hookworm. Even when the analysis is broken down into a younger and an older age group (since *Ascaris* infects primarily younger people), similar results were found: P = 0.989 for ages 20 and under with an odds ratio of 1.006, P = 0.243 for over age 20.

A co-infection scatterplot is shown in Figure 31 to look at the relationship between the levels of infection between the two parasites. The correlation coefficient between hookworm intensity and *Ascaris* intensity of those who are dually infected is 0.562, which is a mildly positive association.

The number of subjects co-infected with hookworm and *Ascaris* and/or *Trichuris* was surprisingly small, due to a relative absence of the latter two nematodes in this area. We were able to show earlier that the hookworm infection was overwhelmingly *N. americanus*, at least in the populations studied during the ummer study. In essence, these populations represent virtually *pure N. americanus* infections, with little contamination by *Ascaris* or *Trichuris*. The high prevalence of hookworm plus the lack of co-infection by other organisms makes this population unique, and ideal for studying *N. americanus* without the confounding effects of *A. duodenale*.

Socioeconomic factors:

Ethnic group comparisons:

The ethnic breakdown of the study group, with very few exceptions, reflects exactly the demarcation between the individual villages used in the study. The residents of Villages 1 and 3 are almost exclusively of Mon descent, while those in Village 2 were likewise of Karen descent. Therefore, it is very difficult to distinguish whether these characteristics (village, ethnicity) are in fact distinct risk factors, or more likely, co-linear variables. It is necessary to look at hookworm epidemiology within and among the different ethnic groups, though realizing that the effects of ethnicity and village will be indistinguishable.

To look at the possible effect of ethnic derivation on susceptibility for hookworm infection, the prevalence and intensity of hookworm infection were compared among the major ethnic groups found in our study. Prevalence studies showed a significant difference in overall prevalence - 59.5% overall of the Mon versus 46.7% of the Karen were infected with hookworm (Pearson chi-square $P = 0.009$).

Figure 32 is visual comparison of the level of hookworm infection between ethnic groups. The Mon had the highest mean intensities of infection, then the Burmese, then the Karen. Those with "missing" ethnic group have a mean intensity between Mon and Burmese. The Mon and the Karen comprised the main ethnic groups studied, and a t-test for independent samples found a significant difference in the intensity of hookworm infection between these (Table 14). Only those positive for infection were included in this comparison.

As state earlier, it is unknown whether being a certain ethnicity is actually a protective factor, or whether these results reflect the general

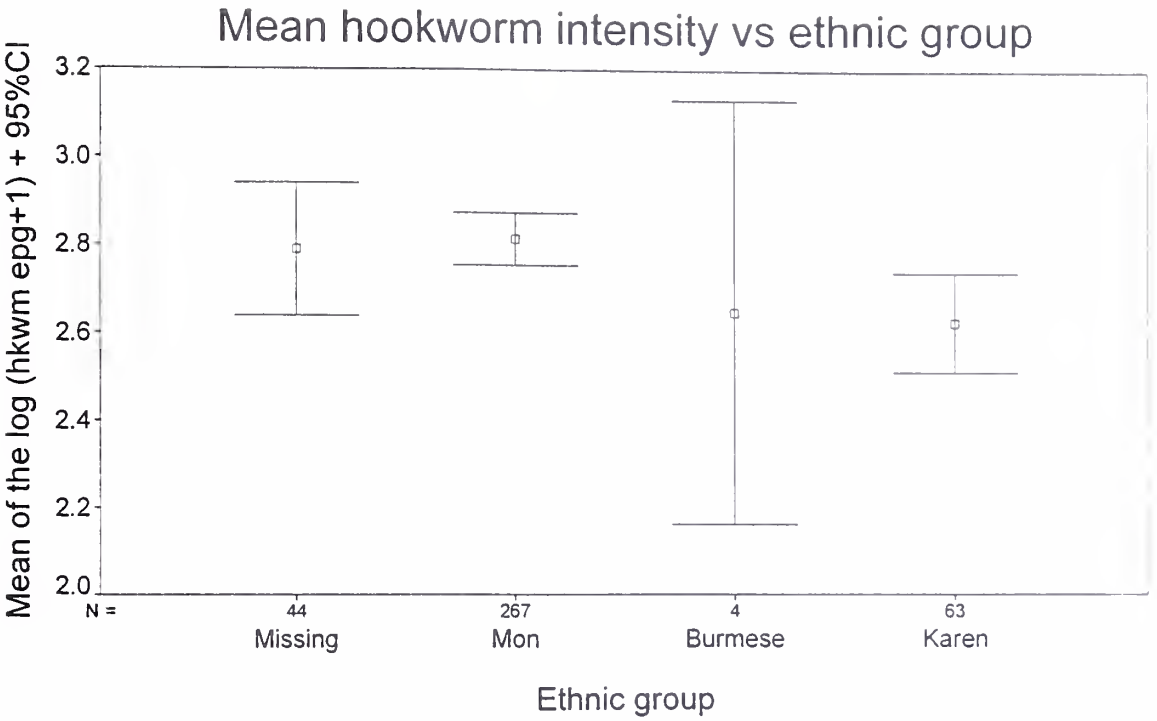


Figure 32. Summer study. Plots comparing overall mean intensity of *N. americanus* infection between ethnic groups. Only those with positive hookworm egg counts were included. Intensity was estimated by eggs per gram stool. Mon and Burmese have comparable mean intensities. Karen have significantly lower levels of infection. N=378

socioeconomic and sanitation differences between the inhabitants of the different villages, since ethnicity is strongly village-dependent.

Table 14. Summer study. T-tests comparing intensity of hookworm infection between the Mon and Karen.

	Levene's Test for Equality of Variances		T-tests for Equality of Means	
	F	*P*	Equal *P*	Unequal *P*
Mon/Karen	1.081	0.299	0.006	0.004

Bathrooms:

Many questions were asked about the toileting facilities available, such as whether a family had a toilet, outhouse, formal hole for elimination, or if the family journeyed out into the woods. If an outhouse/toilet was available, the floor type and the material from which it was constructed were also noted. The Pearson chi-square test found that having a bathroom facility was clearly associated with the person's positive or negative hookworm infection status ($P = 0.033$). There was a significant difference in prevalence between the population with bathrooms and the population without. Risk analysis showed that those without bathroom facilities were at 49% more risk (and maybe up to twice the risk) of acquiring hookworm infection (odds ratio=1.49, 95% CI of 1.01-2.18) than those who had facilities. Phrased another way, having a bathroom was a protective factor.

Once infection was acquired, there seemed to be no difference in the level of infection between the two groups (Table 15).

Table 15. Summer study. T-tests comparing intensity of hookworm infection between those with bathrooms and those without.

	Levene's Test for Equality of Variances		T-tests for Equality of Means	
	F	P	Equal P	Unequal P
bathroom	0.247	0.619	0.836	0.831

The type of bathroom floor was also explored as a risk factor for the transmission of hookworm. In Figure 33 the types of bathroom floors found within each village is graphed. The vast majority of bathroom floors were made of earth in all 3 villages. The highest percentage of people using earth floors was found in Village 1, then Village 3, with the lowest percentage of residents in Village 2 using earth-floor bathrooms. In contrast, Village 2 had the highest percentage of people using bathrooms with cement floors, followed by those in Village 3, with no one in Village 1 using cement floors. A very small proportion in Villages 1 and 3 had floors made of split bamboo. Unlike many native Thais, no tile bathroom floors were reported by any of the families in our study population.

The prevalence of hookworm infection in those with dirt, split bamboo, or cement floors were 56.4%, 71.4%, and 45.2% respectively. A Pearson chi-square test was used to determine whether the type of floor was associated with the prevalence (positive/negative) of hookworm. When dirt vs cement floors were compared, a borderline association (P=0.058) was found. On risk analysis, having dirt floors was also a moderate risk factor, increasing the risk of acquiring hookworm infection by 44% (odds ratio=1.444, 95% CI of 0.90-2.32). However, having bamboo floors in the bathrooms increased the risk significantly, doubling to quadrupling the likelihood of being infected (odds ratio=2.068, range 1.29-4.59). Having cement floors in the bathrooms seemed to have a protective effect (odds ratio=0.63, 95% CI of 0.39-1.03).

The difference in *intensity* of infection (of those who were positively infected) was also found to be important, with persons having bamboo or dirt floors harboring significantly more hookworms (P<0.05) (see Table 16 and Figure 34). It is unknown whether actually having a cement floor in itself is

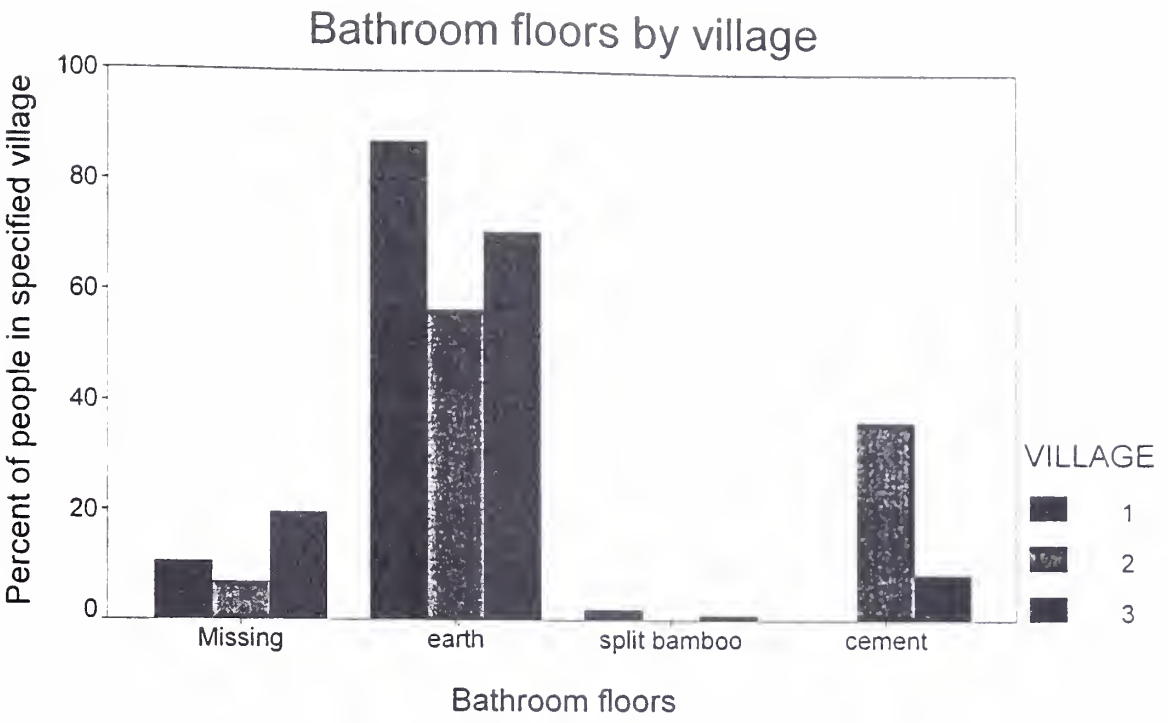


Figure 33. Summer study. Bar graph comparing the types and quantity (measured by % of people using that type) of bathroom floors found in each village.

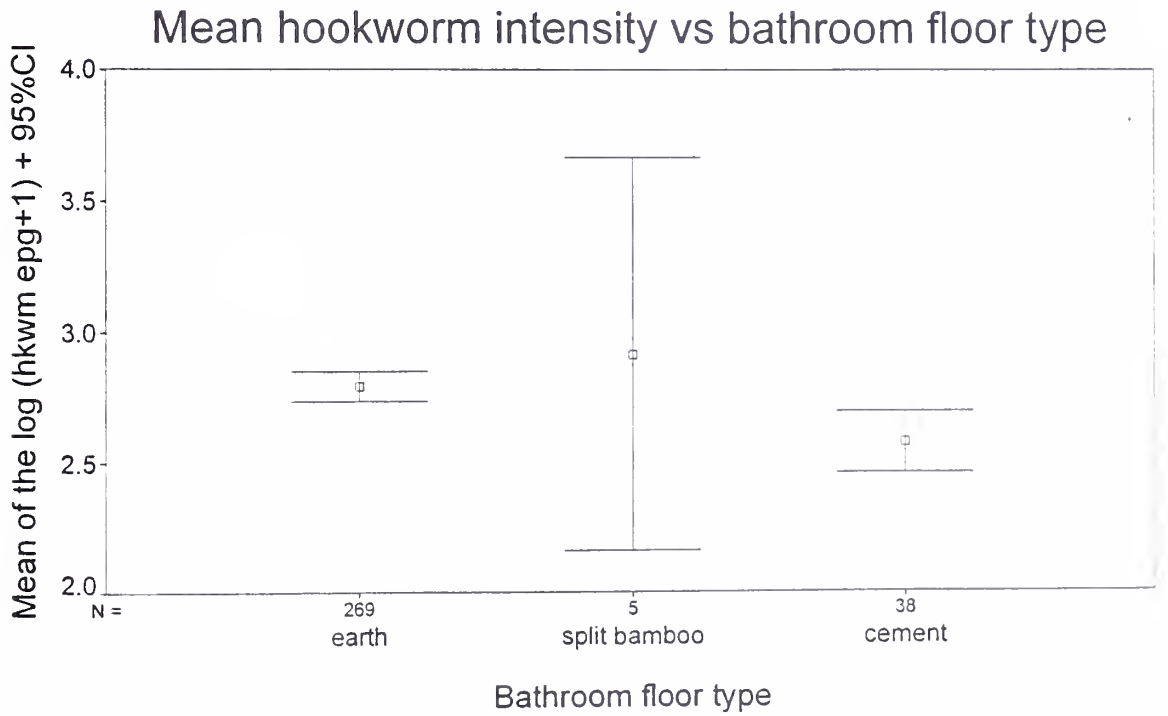


Figure 34. Summer study. Plots comparing overall mean intensity of *N. americanus* infection between the different types of bathroom floors. Only those with positive hookworm egg counts were included. Intensity was estimated by eggs per gram stool. The groups which had either bamboo and dirt bathroom floors had signficantly higher levels of infection, though the variance in the group with bamboo floors is high since the sample size was small. N=378

protective, or whether these results reflect the general socioeconomic and sanitation differences between the inhabitants of the different villages, since the type of bathroom floor is somewhat village-dependent.

To examine further, we looked at Village 2 only in an effort to minimize the variables associated with the other villages. Of the residents of Village 2, 44.4% had bathrooms with cement floors, and the remaining 55.6% had dirt floors. Though the hookworm prevalence (50.0% for dirt, 37.9% for cement) and mean intensity of infection were both slightly higher in the population with dirt floors, the differences were not statistically significant by Pearson chi-square (P=0.150) (for prevalence) and by t-test (for intensity) (Table 16). It seems that the type of bathroom floor was less an important factor in hookworm transmission than the general living conditions within each village.

Table 16. Summer study. T-tests comparing intensity of hookworm infection between those with dirt bathroom floors and those with cement bathroom floors.

	Levene's Test for Equality of Variances		T-tests for Equality of Means	
	F	*P*	Equal *P*	Unequal *P*
All 3 villages	4.258	0.040	0.008	0.002
Village 2 only	1.967	0.166	0.260	0.226

Occupation:

The bargraphs of figures 35 and 36 show the distribution of occupations held by the heads of the household ("father" and "mother"). For the men, day laborers then swidden rice farmers constituted the main occupations held. Most of the women reported being housewives. Of the "fathers", overall hookworm prevalence was 77.0% and did not seem to be related to occupation. Likewise, overall hookworm prevalence was 77.4% for the "mothers" and did not seem to be associated with occupation. For neither sex

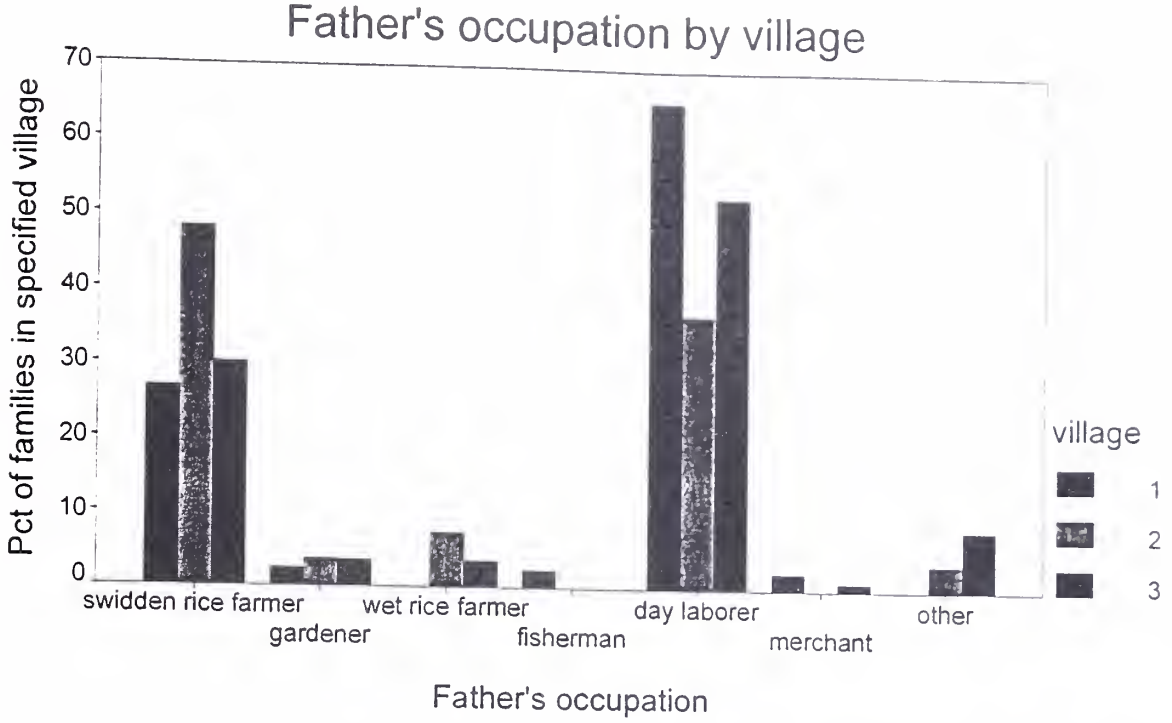


Figure 35. Summer study. Bar graphs comparing father's occupations by village. The two most common occupations in all 3 villages was "day laborer" followed by "swidden rice farmer".

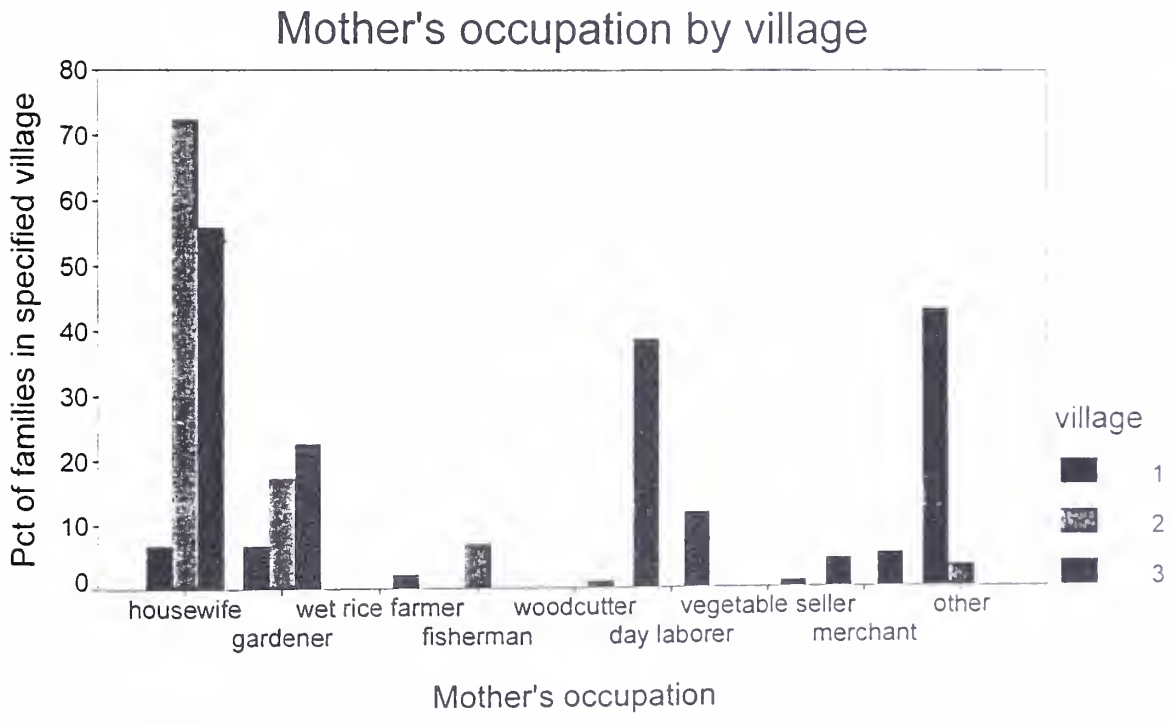


Figure 36. Summer study. Bar graph comparing mother's occupations by village.

was there an association between risk of acquiring hookworm and any specific profession.

Shoe-wearing:

Shoe-wearing as a potential protective/risk factor was eliminated during statistical analysis due to inaccuracies in reporting by the study subjects. Almost all families reported that their members wore shoes all of the time, though it was readily apparent to us in visits to the villages that this was not the case, especially with the children

Vertical transmission:

Both our pilot and summer studies yielded very little evidence for vertical transmission. In sampling by family aggregates, in both studies we were looking for pairs of infected mothers-infants (under the age of 2). The number of mother-infant pairs who were both infected compared to the number of pairs in which one or neither were infected was not statistically significant. In the summer study, we found that the chance of hookworm infection in an infant was identical for those whose mothers were infected and those mothers who were not infected (Pearson Chi-square P=0.495), meaning that having a hookworm-infected mother did not subject the infant to any greater risk of infection (such as in vertical transmission). In fact, further analysis shows the opposite, that hookworm-infected mothers were about 50% less likely to have a positively-infected infant than mothers who are not infected (odds ratio=0.52, 95% CI of 0.06-6.83). However, the sample

sizes were extremely small (only 6 positively-infected infants, and 49 infants total), so the risk is probably similar between both groups of mothers.

Because vertical transmission is thought to occur only with high levels of *A. duodenale* infection in the mother, the lack of statistically significance is probably due to the presence of pure *Necator* infections in this area. Other possibilities for the absence of notable vertical transmission may include the occurrence of arrested development of larvae in the mother without reactivation, or the fact that the pilot study was performed during the dry season in Thailand (January) and intensity of infection at that time is generally low. However, the summer study was performed during the monsoon season, when both prevalence and intensity of infection are usually at their peak.

Those who did not complete the study:

Of the 869 registered in the summer study, 209 did not submit a stool sample. Of those 209, 33 people were away from their homes to find work (23 males, 10 females). The remaining 176 were eligible to submit a stool sample.

As a function of age:

Age distributions are shown for the overall "noncompliant" group (Figure 37). In comparison to the age distribution of the "compliant" group seen much earlier (see Figure 13), the "noncompliant" group contained a much higher proportion of people in early adulthood (early 20's and 30's) and a lower proportion of young children. In fact, while overall "noncompliance" was approximately 20%, the noncompliance hovered as high as 30% in the 16-35 year age range. Therefore, our study was able to include younger children, with a loss of people in early adulthood. This

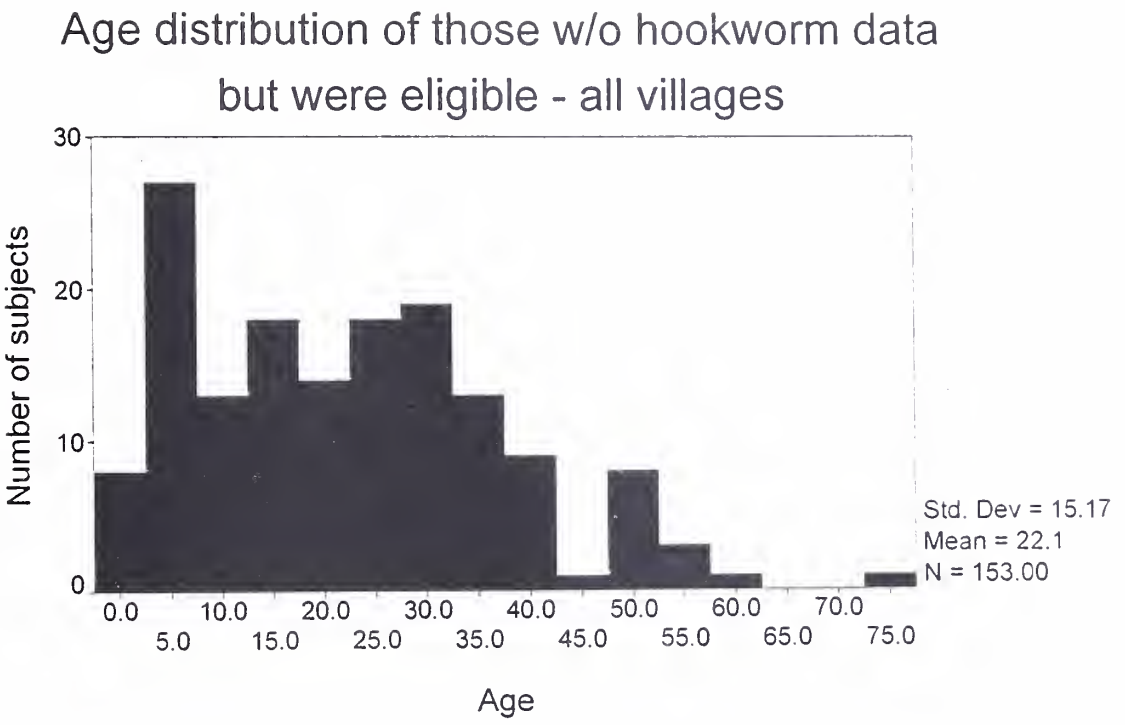


Figure 37. Summer study. Age distribution of those who did not submit stool samples. The age histogram represents the entire "noncompliant" group. N=229

result was not surprising considering that many people left the village during the day for outlying work, a higher fraction of them being the men of the families. As a result, there were approximately twice as many females than males included in the study from the 11-31 year old age range.

As a function of sex:

We noticed a heavy preponderance of females who participated in our study. From the original 869 people registered (49.0% males, 51.0% females), the group of 660 who submitted stool samples was 44.4% male and 55.6% female, while the group which did not submit samples was 63.9% male and 36.1% female. Even after eliminating those who were far away from their homes, this difference in participation between the two sexes is significant (Pearson chi-square P=0.000), and it will be more difficult to apply the results from our studied population to the nonparticipatory population when sex is taken into account.

Table 17. Summer study. Sex breakdown of participation

	N	Males %	Females %
Registered	869	49.0	51.1
Stool sample	660	44.4	55.6
Noncompliant	209	63.9	36.1

Family aggregates

Of those who submitted stool samples for the study, there was a noticeable tendency for either most members of the same family to participate or for no members of the family to contribute. In essence, the participants could be grouped into family aggregates. Intuitively, this could lend bias into our study, since family members share the same activities of daily life, and which make them (as a group) more or less susceptible to acquiring

hookworm infections. This bias could not be eliminated from our calculations.

Discussion

As in many underdeveloped areas, human hookworms and other intestinal nematodes have not been considered a major health threat by either the medical/public health officials or the residents of the small settlements along the Thai/Burmese border. For medical/public health officials, the hookworm problem appears relatively small compared to more immediate and acute health risks, such as malaria and cholera which are common to this area. And, virtually none of the inhabitants of these villages are even aware that these parasites exist on their living and agricultural grounds. This study was important because 1.) it initiated education about the hookworm problem for the first time for the residents of the studied villages, 2.) it was the first determination of the species of hookworm endemic to this region, 3.) it was the first assessment of the levels of hookworm, ascariasis, and trichuriasis for this area, 4.) the risk factors specific to this area and to the behavior of these people were examined, and 5.) all infected participants were treated with anthelminthics after the study was completed.

We have been able to show that the endemic hookworm species here is predominantly and maybe exclusively *Necator americanus*. We have not found any evidence, both morphologically or molecularly, that there is *A. duodenale* or other *Ancylostoma* in this area. This result is not surprising since the predominant species in most of Southeast Asia is *N. americanus* (Carroll and Walker 1990). Mixed species hookworm infections are usually found further north, in the central belt of China. Previous studies of hookworm have tended to combine *N. americanus* and *A. duodenale* into *one* "hookworm" problem, without recognizing the features unique to *N.*

americanus infection or to *A. duodenale* infection. For example, it has been speculated that the intensity of *A. duodenale* infections peaks at an earlier age than *N. americanus* infections. Also, in another example, an immunological response against *N. americanus* may not be as protective against *A. duodenale*. Knowing that pure *Necator americanus* constitutes the hookworm infection in our study, our results give us the opportunity to characterize it as a distinct infection from *A. duodenale*.

Another advantage of our study is that hookworm was the predominant infection in this region, and was usually exclusive of co-infection with *Ascaris* or *Trichuris*. *Strongyloides*, another intestinal nematode which is capable of rapid self-replication, was present but difficult to quantify. Unlike studies performed in China (Xu *et al.* 1995, Wang 1988) and West Bengal India (Nawalinski *et al.* 1978), where there was a very high degree of polyparasitism in the population and within individual hosts, the infection of this region is unusual in its singularity. In individual hosts, there may be a very low degrees of *simultaneous* co-infection by *Ascaris*, *Trichuris*, and hookworm, since prevalence peaks in different age groups for the different parasites (the prevalence of *Ascaris* and *Trichuris* peaks primarily in young children while that of *N. americanus* peaks in early adulthood) (Bundy 1990). However, it is rare for a population as a whole to be infected by only one of the three, and no explanation can be given for the relative absence of the other two geohelminths. The high prevalence of hookworm plus the lack of co-infection by other organisms makes this population unique and ideal for future hookworm investigations and possible vaccine studies.

Not surprisingly, the distribution of *Necator* in our study cohort followed that of a negative binomial distribution. The levels of infection

were aggregated towards the lower intensities, with 65.9% of the hookworm-infected subjects having <1000 eggs per gram stool. On the other end, 14.8% of infected subjects produced 55.5% of the total egg count.

Our studies also concluded that both the prevalence and intensity of *N. americanus* infection do not decrease with increasing age, but in fact plateau at around 26-30 years of age and remain at a constant level throughout the rest of adulthood. As stated earlier in my introduction, many studies on age-prevalence and age-intensity relationships for hookworm have shown conflicting patterns. Some have shown that hookworm levels decrease with increasing age in certain populations, and have attributed these decreases to biological or immunological etiologies. Others studies have given results similar to ours. The constant plateau of the age-prevalence and age-intensity curves can be explained simply by rapid and continual "turnover" of *Necator*, such that constant exposure will maintain the high levels of hookworm infection seen here. Acquired-immunity may still be a strong factor in determining hookworm levels in our study cohort in that, although it did not decrease the levels of hookworm with age, it might have prevented the levels from rising further.

It is possible that our study populations (refugee settlements) have experienced so much immigration and emigration from this area that they have not had the consistent exposure necessary to build a strong immunity against hookworm infection. As stated earlier, the degree of immunity seems to be dependent on the duration of residence in an endemic area (Kloetzel and daSilva 1967). However, the transmigration simply involves crossing the Thai/Burmese border, and all emigrants originated within 50 kilometers of our studies where living and working conditions are almost identical. This short distance plus the similarities in socioeconomic conditions in Myanmar

minimalizes the effects of transmigration on the build-up of acquired-immunity.

Our measurements of hookworm intensity must be cautiously regarded, since in our studies they were performed by calculating the number of eggs per gram of stool rather than using worm expulsion protocols to determine individual worm burdens. As stated earlier, hookworm egg production in geohelminths is density-dependent, with decreased egg production by individual females with increased intestinal crowding (Krupp 1961, Anderson and Schad 1985). Also, the consistency of the stool was not taken into account. Looser stools samples with a higher proportion of water (therefore heavier) may underestimate the actual production of ova since production is measured as eggs per gram of stool. Lastly, egg production within hosts is very variable on a day to day basis, and an infected individual may produce 300 and 1000 eggs per gram stool on two consecutive days (Anderson and Schad 1985). Considering these three factors, the margin of error is wider if "eggs per gram stool" rather than worm expulsion is used as a measurement of infection levels. However, the measurements were still sufficient for examining general trends in the data, which was our purpose.

Sex did not seem to be a major factor in the distribution of *Necator* in our study. However, there was a large disparity between the levels of hookworm among the 3 villages. Clearly, the Karen village (Village 2) had lower prevalences and intensities of hookworm infection than the two Mon villages (Villages 1 and 3). The risk associated with having a certain ethnicity could not be separated from the risk associated with living in a specific village, since these two variables ran together completely. It was very clear that the socioeconomic conditions of Village 2 were at a higher standard than that of Villages 1 and 3. Historically, the Karen group have been

economically more prosperous than the Mon. It is undeniable that a better economic situation is associated with better sanitation, better education and nutrition, and generally a higher standard of living. The residents of Village 2 were as a group more educated, lived in more solidly-constructed homes (less bamboo and thatch, more wood and concrete), and had wider and more paved roads. They generally did not have animals (pigs, chickens, dogs) living under their homes. A much higher proportion of them had outhouses constructed of concrete, which was proven to have a protective effect in hookworm transmission. The local Christian missionary school was located in the village, which treated their students bi-annually with anthelminthic medications. It was stated earlier that "local prevalence and intensity (of hookworm infection) are largely determined by human activities" (Pawlowski *et al.* 1991). The improved sanitary and socioeconomic conditions found in Village 2 have been crucial for reducing the high degree of hookworm transmission found in their neighboring villages.

One of the most important risk factors for hookworm transmission was the presence or absence of a designated outhouse or bathroom, and what the floors of these bathrooms consisted of. The definition of "outhouse" varied among individuals. Oftentimes a family would report that it had a "bathroom", which (on further inspection) was really a deep hole in the ground with a thatched roof and walls constructed around it. We found that having a bathroom or a designated location for defecation was protective against hookworm infection. We found that having bamboo floors increased the risk of hookworm transmission by two- to fourfold (probably because bamboo floors are close to the ground) and dirt floors by twofold. Cement floors seemed to have a protective effect, and almost all of these were found in Village 2.

We were not able to find any evidence for vertical transmission by examining the number of mother-infant pairs. We found that infants of mothers without hookworm infections are just as likely (if not more) to be infected as those infants with infected mothers. This argues against the hypothesis that infants who have infected mothers have a greater chance of themselves being infected via vertical transmission. Also, of those infants who were infected, there was no difference in their levels of infection regardless of whether their mother was infected or not. There are ethical issues involved in trying to obtain colostrum from infected mothers to search for hookworm larvae, since colostrum is widely known to contain immunoglobulins and other factors protective to the nursing infant. Transmammary transmission is theorized to occur only in *Ancylostoma*, but we know that *Necator* is the predominant species here. Our results cannot disprove the existence of vertical transmission, other than to say that we did not see any evidence in this study for it. One can say that our results support the thought that vertical transmission, if it exists at all, takes place only in the *Ancylostoma* species.

Lastly, some thought must be given to the biases of our study, and to those who were asked but chose not to participate. First, the age of our studied population was heavily weighted towards the younger age groups. This effect was exacerbated by the fact that many young adults, usually from the age of 16 to 30 years, traveled away from the village during the day for work, and therefore did not submit a stool sample. A higher proportion of these workers were the men of the family, and in the 16-30 age range the number of female participants nearly doubled that of males. The girls also outnumbered the boys in the childhood age groups - were the boys simply less cooperative, or is this a matter of village demographics? We also noticed a

tendency of obtaining participation in family units - either almost all family members participated, or none of them. Like many studies of this nature, our population was somewhat self-selected. Another bias arose from the possibility that those with the highest levels of hookworm infection and/or suffering the greatest morbidity needed to stay home from work, and were therefore captured in our sampling while the "healthier" ones were not. We were able to get very large sample sizes (both overall and within each age group), which helped to minimize error.

Only one family refused participation early in the study (refused to register), then there were those who were noncompliant in submitting stool samples. We gave anthelminthics to all participants who were infected with hookworm, *Ascaris*, and/or *Trichuris*. Although single doses of anthelminthics have been shown to dramatically improve growth parameters in children (Stephenson *et al.* 1989a,b), it was only a temporary solution to a much greater, long-standing problem. It was our goal to treat as many infected individuals as possible, since only a few untreated people are needed to re-expose an entire community to the parasite. Periodic administration of medication, improved sanitation, and efforts placed in health education are the only means of permanently eradicating hookworm from these populations. In the meantime, further immunological research will contribute significantly to understanding the diseases caused by hookworms and its related parasites.

References

Anderson RM, May RM. 1985. Herd immunity to helminth infection and implications for parasite control. *Nature*, 315:493-496.

Anderson RM, Medley GF. 1985. Community control of helminth infections in man by mass and selective chemotherapy. *Parasitology*, 90:629-660.

Anderson RM, Schad GA. 1985. Hookworm burdens and faecal egg counts: an analysis of the biological basis of variation. *Trans Royal Soc Trop Med Hyg*, 79:812-825.

Brown RC, Girardeau MHF. 1977. Transmammary passage of *Strongyloides sp.* larvae in the human host. *Am J Trop Med Hyg*, 26(2):215-219.

Bundy DAP. 1990. Is the hookworm just another geohelminth? In: Schad GA, Warren KS (eds) *Hookworm Disease - Current Status and Future Directions*. Taylor & Francis, London, 147-164.

Burke TM, Roberson EL. 1985. Prenatal and lactational transmission of *Toxocara canis* and *Ancylostoma caninum*: experimental infection of the bitch before pregnancy. *Int J Parasitol*, 15(1):71-75.

Carroll SM, Walker JC. Hookworm infection in Southeast Asia, Oceania and Australia. In: Schad GA, Warren KS (eds) *Hookworm Disease - Current Status and Future Directions*. Taylor & Francis, London, 33-43.

Chongsuvivatwong V, Pas-Ong S, Ngoathammatasna W, McNeil D, Vithsupakorn K, Bridhikitti, Jongsuksuntigul P, Jeradit C. 1994. Evaluation of hookworm control program in southern Thailand. *SE Asian J Trop Med Public Health*, 25(4):745-751.

Chongsuvivatwong V, Pas-Ong S, McNeil D, Geater A, Duerawee M. 1996. Predictors for the risk of hookworm infection: experience from endemic villages in southern Thailand. *Trans Royal Soc Trop Med Hyg*, 90:630-633.

Ettling J. 1990. The role of the Rockefeller Foundation in hookworm research and control. In: Schad GA, Warren KS (eds) *Hookworm Disease - Current Status and Future Directions*. Taylor & Francis, London, 3-16.

Gilles HM. 1985. Selective primary health care: strategies for control of disease in the developing world. XVII. Hookworm infection and anemia. *Rev Infect Dis*, 7:111-118.

Hawdon JM. 1996. Differentiation between the human hookworms *Ancylostoma duodenale* and *Necator americanus* usng PCR-RFLP. *J Parasitol*, 82(4), 642-647.

Hawdon JM, Jones BF, Hoffman DR, Hotez PJ. 1996. Cloning and characterization of *Ancylostoma*-secreted protein. *J Biol Chem*, 271(12):6672-6678.

Hinz E. 1985. *Human helminthiases in the Philippines.* Springer-Verlag, Berlin, 189-207.

Hoagland KE, Schad GA. 1978. *Necator americanus* and *Ancylostoma duodenale*: life history parameters and epidemiological implications of two sympatric hookworms on humans. *Exp Parasitol*, 44:36-49.

Hotez PJ. 1989. Hookworm disease in children. *Pediatr Infect Dis J*, 8:516-520.

Hotez PJ. 1995a. Hookworm infections. In: Blaser MJ, Smith PD, Ravdin JI, Greenberg HB, Guerrant RL (eds) *Infections of the Gastrointestinal Tract.* Raven Press, Ltd., New York, 1189-1196.

Hotez PJ. 1995b. Hookworms: *Necator americanus* and *Ancylostoma duodenale*. In: Despommier DD, Gwadz RW, Hotez PJ (eds) *Parasitic Diseases* (3rd edition). Springer-Verlag, New York, 17-24.

Hotez PJ, Pritchard DI. 1995. Hookworm infection. *Scientific American*, 272(6):42-48

Keymer A, Bundy D. 1989. Seventy-five years of solitude. *Nature*, 337:114.

Keymer A, Pagel M. 1990. Predisposition to helminth infection. In: Schad GA, Warren KS (eds) *Hookworm Disease - Current Status and Future Directions*. Taylor & Francis, London, 177-209.

Kloetzel K, daSilva JR. 1967. *Schistosomiasis mansoni* acquired in adulthood: behavior of egg counts and the intradermal test. *Am J Trop Med Hyg*, 16:167-169.

Krupp IM. 1961. Effects of crowding and of superinfection on habitat selection and egg production in *Ancylostoma caninum*. *J Parasit*, 47:957-961.

Little MD, Neal AH, Cline BL, Katz SP. 1983. *Ancylostoma* larva in the muscle fiber of man following cutaneous larva migrans. *Am J Trop Med Hyg*, 32(6):1285-1288.

Maxwell C, Hussain R, Nutman TB, Poindexter RW, Little MD, Schad GA, Ottesen EA. 1987. The clinical and immunologic responses of normal human volunteers to low dose hookworm (*Necator americanus*) infection. *Am J Trop Med Hyg*, 37(1):126-134.

Nawalinski TA, Schad GA. 1974. Arrested development in *Ancylostoma duodenale*: course of a self-induced infection in man. *Am J Trop Med Hyg*, 23(5):895-898.

Nawalinski TA, Schad GA, Chowdhury AB. 1978. Population biology of hookworms in children in rural West Bengal, India. General parasitological observations. *Am J Trop Med Hyg*, 27:1152-1161.

Nwosu ABC. 1981. Human neonatal infections with hookworms in an endemic area - a possible transmmary route. In: *Proceedings of the Fourth International Congress on Parasitology*, Section C:10.

Pawlowski ZS, Schad GA, Stott GJ. 1991. *Hookworm infection and anemia, approaches to prevention and control.* Geneva: World Health Organization.

Pritchard DI, Quinnell RJ, Slater AFG, McKean PG, Dale DDS, Reiko A, Keymer AE. 1990a. The epidemiological significance of the immune response to the cuticular collagen of *Necator americanus*: a preliminary study in the hookworm-endemic area in Papua New Guinea. In: *Acta Tropica*.

Pritchard DI, Quinell RJ, Slater AFG, McKean PG, Dale DDS, Reiko A, Keymer AE. 1990b. Epidemiology and immunology of *Necator americanus* infection in a community in Papua New Guinea: humoral responses to excretory-secretory and cuticular collagen antigens. *Parasitology*, 100:317-326.

Prociv P, Croese J. 1990. Human eosinophilic enteritis caused by dog hookworm *Ancylostoma caninum*. *Lancet*, 335:1299-1302.

Roche M, Layrisse M. 1966. The nature and causes of 'hookworm anaemia'. *Am J Trop Med Hygiene*, 15:1031-1102.

Schad GA. 1990. Hypobiosis and related phenomena in hookworm infection. In: Schad GA, Warren KS (eds) *Hookworm Disease - Current Status and Future Directions*. Taylor & Francis, London, 71-88.

Schad GA, Anderson RM. 1985. Predisposition to hookworm infection in human. *Science*, 228:1537-1540.

Schad GA, Chowdhury AB, Dean CG, Kochar VK, Nawalinski TA, Thomas J, Tonascia JA. 1973. Arrested development in human hookworm infections: an adaptation to a seasonally unfavorable external environment. *Science*, 180:502-504.

Scrimshaw NS. 1991. Iron deficiency. *Sci Amer*, 265:46-52.

Stephenson LS, Latham MC, Kurz KM, Kinoti SN, Brigham H. 1989a. Treatment with a single dose of albendazole improves growth of Kenyan schoolchildren with hookworm, *Trichuris trichiura*, and *Ascaris lumbricoides* infections. *Am J Trop Med Hyg*, 41(1):78-87.

Stephenson LS, Latham MC, Kurz KM, Kinoti SN. 1989b. Single dose metrifonate or praziquantel treatment in Kenyan children. II. Effects on growth in relation to *Schistosoma haematobium* and hookworm egg counts. *Am J Trop Med Hyg*, 41(4):445-453.

Stone WM, Girardeau M. 1968. Transmammary passage of *Ancylostoma caninum* larvae in dogs. *J Parasit*, 54(3):426-429.

Variyam EP, Banwell JG. 1982. Hookworm disease: nutritional implications. *Reviews of Infect Dis*, 4(4):830-835.

Walsh JA, Warren KS. 1979. Selective primary health care: an interim strategy for disease control in developing countries. *New Engl J Med*, 301:967-974.

Wang CI. 1988. Parasitic diarrhoeas in China. *Parasitology Today*, 4(10):284-287.

Warren KS. 1988. Hookworm control. *Lancet*, 15:897-898.

Xiao SH, Ren HN, You JQ, Zhao LD, Li B, Zhang CC. 1994. Effect of albendazole on *Ancylostoma caninum* larvae migrating in the muscles of mice. *Chinese J Parasitol Parasit Dis*, 12(3):214-217.

Xu LQ, Yu SH, Jiang ZX, Yang JL, Lai CQ, Zhang XJ, Zheng CQ. 1995. Soil-transmitted helminthiases: nationwide survey in China. *Bulletin of the World Health Organization*, 73(4):507-513.

Yu SH, Shen WX. 1990. Hookworm infection and disease in China. In: Schad GA, Warren KS (eds) *Hookworm Disease - Current Status and Future Directions*. Taylor & Francis, London, 44-54.

Yu SH, Xu LQ, Jiang ZX. 1994. Special report: nationwide survey of human parasites in China. *Southeast Asian J Trop Med Public Health*, 25:4-10.

Yu SH, Jiang ZX, Xu LQ. 1995. Infantile hookworm disease in China: a review. *Acta Tropica*, 59:265-270.

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